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RECOMBINATION ANALYSIS OF BACTERIAL HEREDITY

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Five years ago, at this Symposium, E. L. Tatum and J. Lederberg first reported some preliminary experiments on genetic recombination in *Escherichia coli* K-12 (1946). This report will review subsequent developments in this research, with special emphasis on the application of recombination analysis in bacteria to problems of general genetic interest.

The standard textbooks of bacteriology have emphasized simple fission to the near exclusion of other modes of bacterial reproduction. This is not surprising in view of the confusing aggregate of conflicting and unconfirmed claims of morphological observations interpreted as sexual processes (reviewed by Bisset, 1950). More authoritative claims of bacterial fusion have appeared (Braun and Elrod, 1946; Stempen and Hutchinson, 1951) but none with the essential concurrence of genetic investigation. On the other hand, as we shall see, there is so far no satisfactory demonstration of the morphological basis of genetic interchange in *E. coli*, but the convergence of these lines of work is imminent.

E. coli is a favored organism for genetic work. It is not dangerously pathogenic: it grows rapidly in simple media in highly dispersed form; it is an infrequent aerial contaminant, and is readily identified. It has a widespread habitat, and a great many strains are easily isolated. In addition, we have a considerable background of biochemical information, and the organism is subject to genetic variation in a variety of readily scored characters such as resistance to viruses and chemicals, fermentative reactions, and nutritional requirements or auxotrophy. These advantages are not so unique, however, as to justify neglect of other organisms, especially if sweeping generalizations on bacterial heredity are to issue.

RECOMBINATION IN *Escherichia coli* K-12

Owing to their rarity, genetic exchanges in *E. coli* populations require selective methods for their demonstration. A preferred technique uses auxotrophic mutants, unable to form colonies on a defined minimal medium. Small numbers of prototrophic (nutritionally non-exacting) cells can be selectively isolated in the presence of a preponderance of auxotrophs by inoculating washed cell suspensions into minimal agar: a single plate can be used to screen as many as 10^9 cells for the presence of a single prototroph. The latter is relevant to recombination insofar as prototrophs, A+B+, form one of the classes of recombinants expected from the exchange of factors between two distinct auxotroph mutants, *viz.*, $A-B+$ and $A+B-$. Recombinant prototrophs are also expected to show various assortments of any additional unselected markers that differentiate the auxotrophically distinct parents.

This design has been successfully applied to several strains of *E. coli*, especially "K-12," isolated from human feces in 1922 and maintained since then at Stanford University as a typical strain for laboratory demonstrations (Tatum and Lederberg, 1947; Lederberg, 1947). Techniques for the isolation of auxotrophic mutants have improved steadily; many of them, including the use of penicillin as a selective agent in minimal medium, are documented elsewhere (Lederberg, 1950b). The mutants have generally been isolated from the survivors of drastic treatments with X-rays, ultraviolet light, nitrogen mustard or other mutagens. To obviate the possible confusion between recombinants and those prototrophs originating by spontaneous reversion, doubly or multiply auxotrophic mutants have been used as the parents in most of the experiments. They were obtained by repeating the mutant-isolation techniques on mutant stocks already established.

It may be worthwhile to mention the details of a typical recombination experiment, if only to point out the simplicity of its practice. As parents, we may take the K-12 derivatives 58-161 and W-1177, which differ as shown in Table 1.

The stocks are maintained on ordinary nutrient agar slants, transferred about once every three months. To initiate a cross, each parent is inoculated into a ten ml broth tube (Difco Penassay or other buffered medium) which is incubated overnight without aeration or agitation. The turbid growth is spun down and the supernatant decanted and replaced by sterile saline. It may be advisable to repeat this process, and to rinse the compacted pellet before redispersing it. The

(As an alternative method, selection based on specific "drug"-resistances, has also been successfully applied, as reported by Lederberg, 1950a.)

TABLE 1. SEGREGATING CHARACTERS IN THE CROSS 58-161 × W-1177

Character	Symbol	58-161	W-1177	
Biotin[1]	B	−	+	selected factors
Methionine[1]	M	−	+	
Threonine[1]	T	+	−	
Leucine[1]	L	+	−	
Thiamin[1]	B_1	+	−	
Lactose[2]	Lac_1	+	−	unselected markers
Maltose[2]	Mal_1	+	−	
Mannitol[2]	Mtl	+	−	
d-Xylose[2]	Xyl	+	−	
Virus Tl; T5[3]	V_1	s	r	
Streptomycin[3]	s	s	r	

[1]Auxotrophy (+ independent; − dependent)
[2]Fermentation
[3]Sensitivity or resistance

The derivation of these stocks is cited in Tatum, 1945 and Lederberg, 1947, 1949. Each character was altered in a single mutational step.

final suspension of washed cells should comprise a reduced volume of two to three ml. The two suspensions are combined. Samples of 0.05 to 0.1 ml are then spread over minimal agar plates, or poured with melted agar. The plates are then incubated (at 30° or 37°C). On each plate a few hundred prototroph colonies appear in 24 to 72 hours, while control plates (58-161 or W-1177 suspensions alone) have invariably remained entirely barren. The prototrophs are then picked for further tests. It is important to keep in mind the usually invisible background of auxotroph parent cells which may interfere with tests for the unselected markers unless the prototrophs are purified by conventional methods. The media used are not at all critical; the formulas in use at Wisconsin are given elsewhere (Lederberg, 1947, 1950b). Fermentation markers are scored by inoculating EMB (eosin methylene blue peptone) agar containing one per cent sugar. Resistance is scored by cross-streaking a suspension of the bacteria against a loopful streak of the virus or chemical.

This cross has been studied extensively in several laboratories besides our own with concordant results. Among the prototrophs, each of the possible assortments of the unselected markers may be found if a reasonably large sample is studied. The reassortment of unselected markers is the most cogent evidence that the prototrophs arise by recombination, rather than by any artifact. The different combinations do not occur with equal frequencies by any means, pointing to a linkage system which is discussed further below.

This simple experiment demonstrates genetic interaction between the parental bacteria, but leaves open several questions as to its mechanism. If we confine our discussion to effects based upon material exchanges (excluding *a priori* mitogenetic rays and the like!) two contrasting hypotheses exhaust the possibilities: (A) one or both the parents release chemically definable substances which transform the other into prototrophs; (B) a fusion of organized elements of cellular origin is involved. Hypothesis (A) parallels the interpretation given to type transformation experiments with preparations containing polymerized DNA from pneumococci or influenza bacilli (McCarty, Taylor and Avery, 1946; Alexander and Leidy, 1951). Hypothesis (B) entails a sexual process with attendant implications of processes analogous to fertilization-reduction cycles. At the outset, it should be made clear that a final decision between these hypotheses must wait upon a positive chemical characterization or morphological demonstration of the agents of recombination. This is not yet at hand, but there are many genetic and negative physical observations each of which, in our opinion, weighs compellingly in favor of a sexual process in this bacterium. The main difficulty in the way of more direct physical evidence on this question is the relatively low rate of recombination, for the yield of prototrophs is only about 10^{-6} of the auxotroph cells inoculated.

Experiments designed to find support for "transformation" have failed to implicate any unit other than the intact cells of the two parents. The prototroph-forming ("transforming") activity of cultures in broth is quantitatively sedimented with the cells in the centrifuge, and no such activity can be found in the supernatants passed through bacterial filters, nor in preparations prepared from autolysates or other cell-free preparations. Davis (1950b) has reported that repeated flushing across a filter of the medium shared by two parents resulted in no transfer of activity. The stratification of the two parents in contiguous layers of minimal agar nearly eliminates their interaction. Finally, the addition of desoxyribonuclease to interacting cells has no effect, in contrast to its destruction of the transforming factors of the pneumococcus and of *Hemophilus*. We are led by these experiments to con-

clude that the agent of recombination is either cellular, or very labile except in intimate association with cells.

The genetic properties of the recombination system agree with this conclusion. There is no obvious limit to the number of factors which can be exchanged at one time; in 58-161 × W-1177, there are no less than six unselected markers (7, including B_1—on thiamin agar), all 64 combinations of which occur among the prototrophs. Each of a considerable number of other markers tested in other crosses has segregated in similar fashion. By contrast, the transformation experiments so far published have involved one character at a time.

Further experiments involving the mixture of three well-marked stocks have shown that recombination is limited to factors differentiating single pairs of cells, and there is no pooling of genes from more than two cells at a time (no *ménage à trois*). Finally, non-disjunctional exceptions have been found (Lederberg, 1949) in which the parental genomes are associated in heterozygous cells, which segregate at occasional fissions to unmask the recessive components. This interpretation is strengthened by Zelle's single cell pedigrees which independently rule out the possibility that the "heterozygotes" are associations of intact cells (Zelle and Lederberg, 1951).

The simplest picture which encompasses present information supposes a life cycle similar to that of *Aspergillus* or *Zygosaccharomyces*: a haploid vegetative phase (inferred from segregation in the f-1), an infrequent and usually transient diploid phase following upon fertilization, and no evidence of heterothallic restrictions on compatibility. For the moment, I am inclined to the view that fertilization is isogamous, and follows the fusion of undifferentiated vegetative cells, but the possibility of gametic specialization is not entirely out of the question. But as stated before, a morphological concordance is needed and *E. coli* is perhaps not the preferred organism for this purpose.

The efficiency of recombination between cells mixed for the first time on minimal agar plates may call for some comment. There is evidently a limited amount of residual growth of the auxotroph inocula, undoubtedly assisted by syntrophic stimulation. The prototrophs probably result then from the interpenetration of microcolonies rather than individual cell-to-cell contacts. Any analysis of the kinetics or physiology of bacterial recombination should define the proximate conditions of cell contacts more closely than has so far been achieved (e.g., Clark, *et al.*, 1950). For example, the non-specific formation of small mixed clumps of cells would be expected to encourage their later sexual association in the face of their immobilization by the physical restraints imposed by solid medium. This is a reasonable interpretation of Nelson's (1951) findings that the yield of prototrophs increases with the time during which the parents are shaken together in saline suspensions before plating.

Formal Genetics of Haploids

Some hint of the genetic structure of *E. coli* is given by the segregation of markers among prototrophs, but the necessity for employing a selective method introduces some difficulties. The exclusion of non-prototrophs precludes the isolation of complementary segregants as a class or as multiple products of single meioses. This disallows some necessary checks on the mathematical regularity of segregation, and vitiates the random recovery of sexual progeny necessary for statistically mendelian behavior. Furthermore, we have no direct way of knowing what proportion of the zygotes succeed in producing a detectable prototroph recombinant. This difficulty is very well illustrated in attempts to unravel the linkage system that generates the empirical rules for the segregation of the markers in such crosses, as 58-161 × W-1177.

When crosses are carried out according to the protocol described in the first section of this paper, the potential zygotes are immobilized on the agar, so that their genetic products are also localized. With very few exceptions, the individual prototroph colonies are internally homogeneous with respect to segregating markers, and show no signs of subsequent segregation either under ordinary culture or single cell isolation (Zelle, unpublished; see Lederberg, 1947). However, the different colonies vary from one to the other: segregation occurs when the prototroph colony is initiated. If a reasonable proportion of zygotes produce prototrophs, we could also conclude that the intermediate diploid phase is transient, does not proliferate as such, and resembles the many other haplobiontic thallophytes. We might also conclude, but less surely, that diploid fusion *nuclei* occur individually, despite the cytological evidence of several nuclei per cell. Whether this means anisogamy, or that cell fusion occurs rather more frequently than karyogamy, or something else, we cannot say. The recombinant prototrophs are culturally and genetically indistinguishable (except for their

inherited markers) from the typical parental cultures. At least three other groups of workers working with the cross 58-161 × W-1177 have obtained detailed results quite consistent with ours (Table 5) on the segregation of the markers among prototrophs (Cavalli, 1950; Newcombe and Nyholm, 1950; Gordon Allen, unpub.). Clearly, the frequencies with which the different types appear are sufficiently stable to justify a search for definite rules of segregation. Further support for regarding these mutations as indifferent markers of the genetic mechanism comes from "reverse crosses." The same differential markers are introduced, but with the alternate parent: for example, $B-M-Lac+V_1^r \times T-L-B_1-Lac-V_1^s$ is compared with $B-M-Lac-V_1^s \times T-L-B_1-Lac+V_1^r$. The segregation frequencies of *Lac* and V_1 are inverted by this reversal; in other words, the occurrence of a given allele among the prototrophs is regulated not by the nature of the allele, but by its parental coupling.

To apply this test, recurrent mutations in the two parental (*BM* and TLB_1) stocks must be obtained and this is not always easy. However, it has been applied successfully to markers at the following loci: Lac_1, Mal_1, V_1, V_6, S. Table 2 illustrates the experimental concordance of bacterial segregations to a generalized definition of mendelism: "The gametic frequencies are invariant in respect of any gene substitution applied systematically to the genic content of an organism and of the gametes it produces" (Fisher, 1947). Here, of course, we must read f-1 haploid progeny for gametes. (The following notation is used to avoid confusion between haploid and diploid generations: p-1 × p-1 (n) ⟶ F-1 (2n) ⟶ f-1 (n). f-1 × f-1 ⟶ F-2 (2n) ⟶ f-2, etc. These are designated as p-1, f-1 crosses, etc.)

With the data reproduced in Tables 2, 3, and 4, an attempt has been made to map certain factors, and especially to determine whether the concept of linearity can be unambiguously tested. We proceed from the assumption that segregation is regular, but that our observations are confined to the prototroph set of recombinants. The distribution of unselected markers will be then controlled exclusively by their linkage relationships to the selected, nutritional markers. Thus we infer that *the scarcity of one allele of a marker gene signifies a linkage to the auxotrophic factors originally coupled with it in one of the parents.*

TABLE 2. REVERSE CROSSES INVOLVING PERMUTATIONS OF *Lac* AND V_1

Parents*		Prototrophs: Percentage Distribution‡				No. tests
$B-M-T+L+$	$B+M+T-L-$	$B+M+T+L+$				
Lac V_1	*Lac* V_1	$Lac-V_1^r$	$Lac-V_1^s$	$Lac+V_1^r$	$Lac+V_1^s$	
+ r	− s	B 42.7	C 23.2	A 32.5	ABC 1.6	2013
+ s	− r	C 34.6	B 42.5	ABC 2.6	A 20.3	696
− r	+ s	A 25.1	ABC 2.5	B 47.7	C 24.7	518
+ r	− r†	B or C 79.5	0	A 20.5	0	161
− r	− s†	—134 *Lac*−; V_1 not scored.—				

*B_1 +/− was also segregating, but has no interaction with other factors. The data given here were therefore pooled. Adapted from Table 5, (Lederberg, 1947).

†Allelism tests.

‡The letters A, B, C, and ABC refer to single and triple crossovers in the regions $[BM]-Lac$; $Lac-V_1$; and $V_1-[TL]$, respectively.

For example, the relative paucity of V_1^r among prototrophs ($B+M+T+L+$) from the cross $B-M-V_1^s \times T-L-V_1^r$ suggests that V_1 is linked to *T* or *L*. Since these segregate dependently, V_1 is linked to both, but the order is indeterminate. When the appropriate growth factor is added to the plating medium, individual nutritional loci may be treated as unselected markers in the same way. Thus we may interpret Table 3 to specify the arrangements: $B_1-[B\text{-}M]$ and $[T\text{-}L]$. Insofar as a re-

TABLE 3. RELATIVE FREQUENCY OF MONOAUXOTROPH RECOMBINANTS

$B-M-T+L+B_1+ \times B+M+T-L-B_1-$

Monoauxotroph Class	$B-$	$T-$	$L-$	B_1-
Ratio to Prototrophs	0.17	0.24	0.10	9.88
Number of Tests	70	46	56	87

The cross was conducted on minimal agar with single growth factor supplements. The recombinants were then classified as prototrophs or monoauxotrophs, and their relative frequency recorded. *M*− is not recorded, as this test cannot be conducted on methionine medium with B− as the only selective marker for the $B-M-$ parent. Adapted from Table 4. Lederberg, 1947.

combination between [*B-M*] and [*T-L*] is indispensable to the occurrence of a detectable prototroph, we can say nothing of their connection so far. Upon these coordinates the data of Table 2 enable us to map the *Lac* and V_1 factors. The individual segregation frequencies show: B_1–[*B-M*]–*Lac* and V_1–[*T-L*] respectively. The interaction between *Lac* and V_1 (i.e., the marked deficiency in $Lac+V_1^R$ from *BM Lac*+ V_1^s × *TL Lac*– V_1^r) gives us: B_1–[*B-M*]–*Lac*–V_1–[*T-L*], where the order of factors in [ ] is still indeterminate. So far, we have had no

TABLE 4. LINKAGE OF *Lac*, V_1 AND V_6

B–M–T+L+B$_1$*+Lac+*$V_1^r V_6^s$ × *B+M+T–L–B*$_1$*–Lac–*$V_1^s V_6^r$

Prototrophs	*Lac*:	–	–	–	–	+	+	+	+
B+M+T+L+	V_1 :	r	s	r	s	r	s	r	s
	V_6 :	r	r	s	s	r	r	s	s
	% :	43	33	1.7	0	4.6	1.1	15	1.7
Postulated crossover		c	d	abc	abd	b	bcd	a	acd

BM	V_6	*Lac*	V_1	*TL*
–	s	+	r	+
+	R	–	s	–
	a	b	c	d

As in Table 1, B_1+ and B_1– data are pooled. Total tests numbered 176. Adapted from Table 6, Lederberg, 1947.

test of linearity, for each experimental fact was accommodated by adding an independent element to the scheme. The first opportunity for such a test arose with the placement of V_6 (resistance to phage T6), as shown in Table 4. The segregation ratio of V_6 places it near [*BM*], and the test of linearity now requires that it be linked either to B_1 on the left, or *Lac* to the right. The latter appears to be justified, for there is only about six per cent recombination between *Lac* and V_6. There are, however, an unusual number of complex recombinants that would have to be interpreted as multiple crossovers. Further linkage studies have been somewhat hampered by the paucity of suitable markers, especially in the vicinity of V_1.

The most concrete difficulty with the scheme developed with attempts to map the *Mal* factor, and subsequently, *S*. In 58-161 × W-1177 the frequency of *Mal*+ is about 15 per cent, placing it near [*BM*] (Table 5a). It is not closely linked either to B_1 or *B*, for the addition of biotin and thiamin to the medium does not appreciably alter the 15 per cent figure. Therefore, if the data

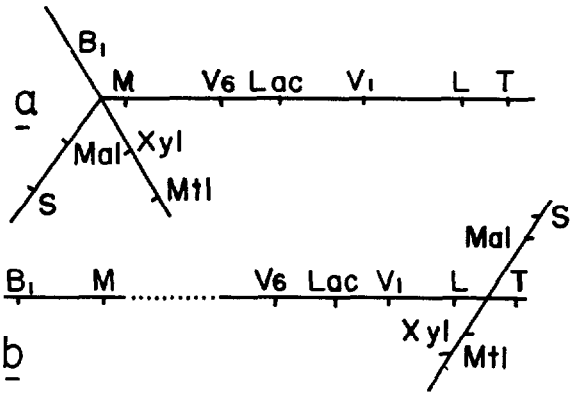


FIG. 1. Schematic representation of linkage data. a. 58-161 × W-1177. b. 58-161 × F-1, W-1177 type. (Compare with Table 5.) This diagram is purely formal and does not imply a true branched chromosome.

are to be fitted to a linear scheme we would expect *Mal* to appear between [*BM*] and *Lac*. However, the segregations of *Mal* and *Lac* are uncorrelated except that the frequency of *Mal*+ is somewhat higher among the *Lac*+ than the *Lac*–.

Consideration of the other markers of W-1177 multiplies the difficulties. *S* shows what appears to be a straightforward linkage to *Mal*, most of the prototrophs being either *Mal*+ S^r or *Mal* – S^s (these data were collected in part by M. Doudoroff). Similarly, *Xyl* and *Mtl* appear to show simple linkage to each other. However, although the segregation ratio for *Mtl*+ is also of the order of ten or 15 per cent, it is not clearly linked either to B_1, or to *Lac*, or to *Mal*, but there are statistically significant interactions, especially with *Mal*.

In a purely formalistic way, these data could be represented in terms of a 4-armed linkage group, Figure 1a, without supposing for a moment that this must represent the physical situation. This recalls the branched chromosome representation (Hamlett, 1926) of translocation heterozygotes in *Drosophila* before the cytogenetics of this situation was well understood. Newcombe and Nyholm (1950a) have, however, interpreted or described the deviations in linkage behavior as due to "negative interference." These authors place *Mal*, *S*, *Xyl*, etc., to the left of [*BM*] but have not studied the anomalous behavior of B_1. Since we are armed exclusively with genetic data in *E. coli*, and since the cornerstone of *genetic* linearity is the lower frequency of multiple compared to single crossovers in small regions, this discussion may be somewhat premature. For this reason, our accumulated data on these segregations have been held in abeyance

until they could be correlated with other information to be discussed presently. We feel that the premises on which the mapping from prototroph data is founded must be reexamined and verified before the problem can be settled.

Our suspicions concerning the placement of *Mal* were deepened by the behavior of nondisjunctional exceptions, the first of which was encountered about three years ago. The principal deviation was the finding that each of the several hundred diploid heterozygotes obtained from crosses of one nondisjunctional line, and almost all others, proved to be haplogenic for *Mal* (and *S*), although digenic for most other markers. In crosses of the type form *B-M-Het* × W-1177, or 58-161 × W-1177 *Het* (*Het* is the factor that promotes nondisjunction), about four-fifths of the heterozygous prototrophs are *Mal*– (i.e., the same as the haploid prototrophs in this or the type cross). This suggests that the segregation frequency of *Mal* is determined not by auxotrophic linkage, but by another process which eliminates a segmental homologue encompassing the *Mal* and *S* loci. In this way also, the inconsistencies in the apparent linkage of *Mal*-[*BM*], and of [*BM*] to B_1 and to *Lac*, may be obviated. The details of this obviously highly aberrant behavior are far from clear. A further indication of structural heterozygosity may be seen in f-1 and f-2 crosses, in which haploid segregants from diploid nondisjunctions are used as parents. When *B-M*-f-1's were crossed with *T-L-*B_1-*p-1* stocks carrying the appropriate complementary markers, no alteration of segregation ratios was observed. However, each of four or five *T-L-*B_1- segregants studied in the same way gave highly perturbed ratios when crossed either with p-1 or f-1 *B-M-* stocks. Table 5 presents a comparison of crosses of p-1 B-M- with W-1177 and a phenotypically similar W-1635, extracted from the heterozygote H-267 (W-67 × W-1177), mentioned later. All of the markers except B_1 behave in the latter cross as if they were linked to [*TL*], but with the same lack of interaction as before, so that we have (again formalistically) a multiarmed configuration as in Figure 1b.

The main point is that we have a derived stock whose apparent linkage relationships are entirely unique. In any plant or animal this would be *prima facie* evidence of a chromosome aberration, and this is one working hypothesis. However, we cannot say whether W-1635 or W-1177 (if either) is structurally homologous with 58-161 or the original K-12.

TABLE 5. SEGREGATION DATA IN MULTIPOINT TESTS

	Par																				Par																A	B
Lac	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	28	85
Mal	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	21	84
S	r	r	r	r	s	s	s	s	r	r	r	r	s	s	s	s	s	r	r	r	r	s	s	s	s	r	r	r	r	s	s	s	s	s	s	s	15	72
Mtl	+	+	–	–	+	+	–	–	+	+	–	–	+	+	–	–	+	+	+	–	–	+	+	–	–	+	+	–	–	+	+	–	–	+	+	–	17	73
V_1	r	s	r	s	r	s	r	s	r	s	r	s	r	s	r	s	r	s	r	s	r	s	r	s	r	s	r	s	r	s	r	s	r	s	r	s	67	88
A. p-1 × p-1		1	3	4					3		22	36			1	2		1		1	1	9	1		1	1	1	11					1						
B. p-1 × f-1	1			1	3	6		2		1	1						2	13			4	45		7	1	5		3		2		3						

The figures represent the numbers of different recombinant types found in 100 tests of randomly isolated prototrophs from crosses of the form of 58-161 × W-1177. Series A describes just this cross; B shows the deviations found when a phenotypically similar f-1 segregant is substituted for W-1177. These data are presented to illustrate typical patterns. For more detailed linkage studies, rarer subtypes such as *Mal*+ or S^r are isolated selectively for large scale testing (compare Cavalli, 1950; Newcombe and Nyholm, 1950a and b). Columns A and B summarize the per cent + or s for each marker in the two crosses.

With some effort, di-auxotrophic recombinants can be recovered (e.g., Table 2 of Tatum and Lederberg, 1947) by conducting the crosses on appropriate media. Crosses of the form *M-P-* × *T-L-Lac-Mal-* etc., have been made on minimal medium supplemented either with methionine + leucine, or proline + threonine. A small proportion of the nutritional selections are *M-L-* or *P-T-* recombinations, respectively, most of the colonies being prototroph recombinants, or monauxotroph recombinants or reversions. Since the *M-L-* and *P-T-* are complements, regular segregation would require that the segregation ratios for unselected markers be inverted from one to the other set. In preliminary experiments, Miss Phyllis Fried has found *Lac*+/– to conform to this expectation, but both sets were almost uniformly *Mal*- like the prototrophs. If these findings

are correct, we can be certain that the segregation of *Mal* is determined otherwise than by linkage to [*BM*]. The *M-P-* stock used in these experiments was prepared by Gordon Allen for a somewhat similar purpose: to look for the complementary auxotrophs as segregants from single zygotes. He was unable to find conclusive evidence on the question owing to technical difficulties, but incidentally accumulated segregation data in agreement with those just mentioned.

The rule was stated earlier that individual prototrophs were generally pure for segregating markers. In addition to nondisjunctional exceptions a second type of exception has been found in what I shall call "twin prototrophs." These are detected most readily as bisectored prototroph colonies from crosses on a synthetic indicator medium ("EMS") upon which one fermentative character of a colony can be read by inspection. One or two per cent of the prototrophs on EMS-maltose medium are duplex. At first thought, these might be either coincidences of zygotes, or sister segregants from a single zygote following four-strand multiple crossing-over. Both these notions are ruled out by the distribution of other markers to the twins. In particular, the alleles for *Lac* and V_1 are almost always the same in the two gemini, even when the combination is the rare $Lac+V_1^r$. V_6 also follows *Lac; S* follows *Mal*, and *Xyl* and *Mtl* show no clear preference. This result could be interpreted in terms of three chiasmata, two of them (involving *Lac*-V_1) showing strong negative chromosome and positive chromatid-interference, but this seems rather farfetched. It may be more reasonable that the *Lac*-V_1 character of the twins is determined by a single crossover event, but that reduction for *Mal* and *S* was delayed for one fission. This would be the converse of the aberrant nondisjunctions which are reduced for *Mal* and *S*, but in which reduction for *Lac*, etc., may be delayed indefinitely.

Formal Genetics: Nondisjunctional Exceptions

Throughout the preceding discussion the diploid zygote has been an unseen logical inference deduced from the occurrence and patterns of recombination, rather than a tangible reality. Many of the assumptions made to provide a framework for further experimentation could be tested much more directly if the zygotes could be collected and characterized as such, and then allowed to segregate. Many early experiments (1947) were carried out to try to induce nondisjunction or persistence of the diploid phase by heat shocks, variations in medium, irradiation, and c-mitotic chemicals, but none were successful or even encouraging. On one occasion, in 1948, however, one of our strains itself set out on this experiment, and the larger part of our attention since then has been directed to the analysis of diploid behavior.

The exception was detected in a curious manner worth recounting. The genetics of phage resistance was being studied, and a number of mutants resistant to T1 were collected. One of the mutations, designated V_1^p, was partially resistant to T1 and T5, and was tested to see if it carried an intermediate allele of V_1^r, which is immune to both these phages. The V_1^p mutant occurring in 58-161 (*B-M-*) was therefore crossed with a *T-L-*B_1-V_1^r *Lac*-stock (Y-64), and 200 prototrophs were tested on EMS medium for their reaction to T1. Of these, 199 segregated V_1^p and V_1^r; a single prototroph scored V_1^s. Insofar as the decision between a series of multiple alleles at V_1 as against two linked loci rested upon this single apparent recombinant, it was retested against T1 on EMB (complete) medium. This time it showed a peculiar reaction, as if it were a mixture of sensitive, partially resistant and resistant. When the culture was streaked out on EMB lactose medium, it became apparent that several types were splitting off, the most obvious being $Lac-V_1^r$ and $Lac+V_1^p$ like the parents. When these were purified and tested further, it was found that many of them were auxotrophic for various combinations of *B, M, T, L* and B_1, although they had been derived directly from colonies growing well on synthetic (EMS) medium. On this medium, there is little overt evidence of segregation, for auxotrophic segregants are suppressed. After 15 single colony transfers on EMS lactose, the culture still segregated on EMB medium. From this, and the interaction of V_1^r with V_1^p to give the V_1^s reaction, the possibility of an extra-cellular association of the two parents was rejected, and either a heterokaryon or heterozygote was postulated. The occurrence of new combinations among segregated colonies supports the latter, although it is quite likely that a heterokaryon intervenes temporarily between nuclear and cell reduction. The appearance of segregating variegated (designated *Lacv, Mtlv*, etc.) colonies on EMB medium is quite characteristic, as shown in Figure 2. Any lingering doubt that the variegation might result from the sticking together of cells of the two parents should be dispelled completely by Zelle's single

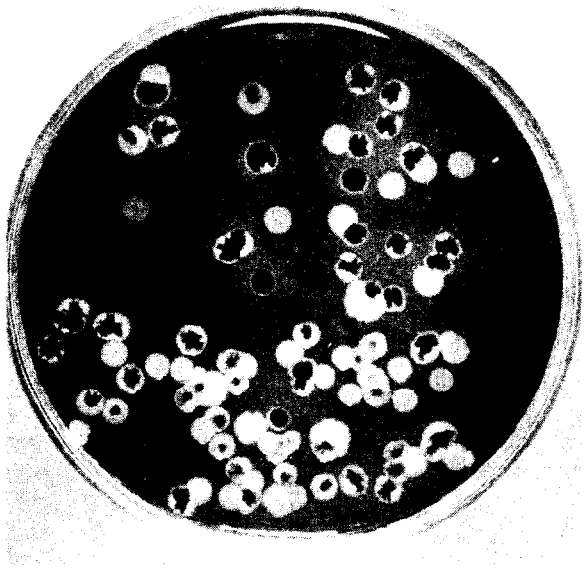


FIG. 2. Segregation of heterozygous diploid cells to form variegated colonies: *Mal*+/– on EMB maltose agar.

cell pedigrees, in which heterozygous cells were permitted to divide repeatedly under close microscopic observation, while the entire pedigree was isolated with the micromanipulator (Zelle and Lederberg, 1951). Two such pedigrees (typical of a score of them) are shown in Figure 3. In 3a, every viable cell produced a variegated colony; in 3b, the other pedigree, a segregation occurred during an early fission, giving a clone of eight haploid cells. As a rule, the segregants occurred individually, not as complementary pairs (Zelle and Lederberg, unpublished). This may be attributed to a dissynchrony between nuclear and cell-division, and to the postulated haplolethality of the segmental deficiency discussed below. A rather high proportion of inviable cells was observed, but this genetic basis remains to be proven.

The first nondisjunction found (H-1) was not very well suited for segregation studies owing to the paucity of easily scored segregating markers, but it quickly became apparent that alternative factors did not occur with equal frequency. Among segregants isolated at random from complete medium, the majority were $M+Lac-V_1^rT-L-B_1-$. The other parental type, $M-Lac+V_1^p$ occurred less frequently, but prototrophs and the previously elusive polyauxotroph recombination $M-T-L-B_1-$ were also found. Once established, the segregants remained perfectly stable, and segregation for different factors occurred concurrently. This, together with the fact that instability was confined to the factors which differentiated the parents, leaves little doubt that the genetic instability of these cultures results from occasional segregations of hybrid cells.

Some of the *B-M-* and *T-L-B*$_1$- segregants of H-1 were preserved for use in f-1 crosses, particularly W-478 (*B–M–Lac+Het*) and W-477

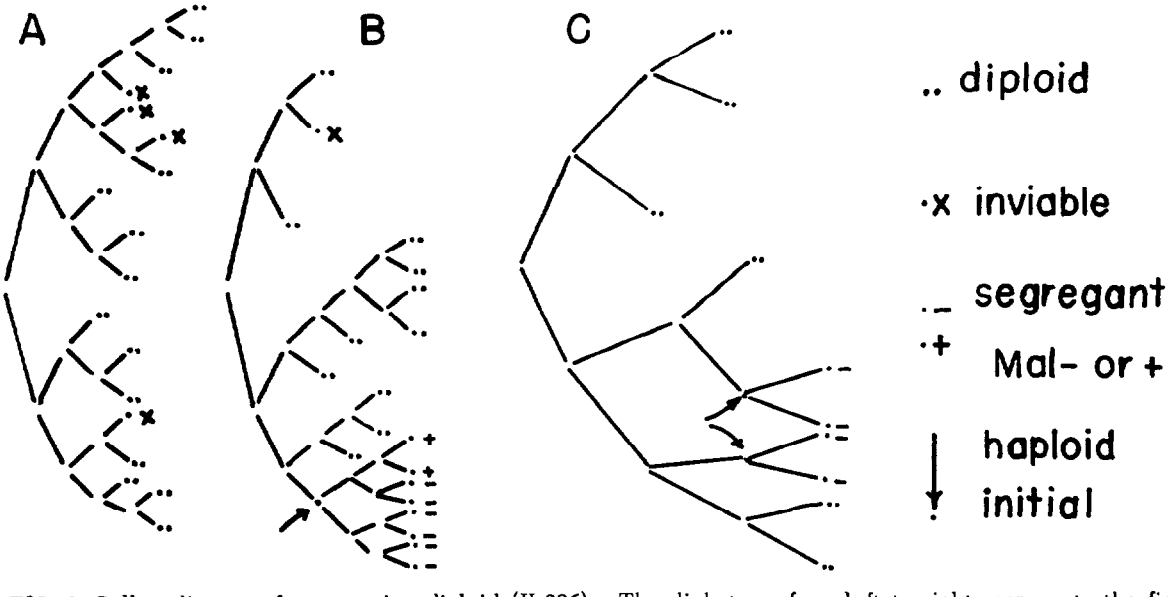


FIG. 3. Cell pedigrees of segregating diploid (H-226). The dichotomy from left to right represents the fissions of a clone under microscopic observation. The fission products were separated and the terminal cells isolated with the aid of a micromanipulator (adapted from Zelle and Lederberg, 1951).

TABLE 6. NONDISJUNCTION PROTOTROPHS ISOLATED FROM
B−M−V_6^r S^r Het × *T−L−B_1−Lac_1−Mal_1−Xyl−Mtl−*

A: *Mtl v* Isolations (39)						B: *Lac v* Isolations (28)			
No.	*Lac*	V_6	*S*	*Mal*	*Xyl*	No.	*Mtl*	*Xyl*	*Mal*
19	v*	v*	s	−	v	12	v	v	−
6	−	s	s	−	v	4	+	+	+
3	v	v	r	+	+	3	−	−	−
2	v	v	s	−	−	3	v	−	−
2	v	v	s	+	+	3	+	+	−
2	−	v	s	−	v	1	−	v	−
1	v	v	r	+	v	1	+	v	+
1	+	r	r	+	+	1	−	+	+
1	v	r	s	−	v				
1	−	v	r	+	v				
1	mucoid: unclassifiable								

*v refers to heterozygous condition, whether +/−, or r/s. No *Malv* were recovered in several hundred tests of *Mal*+ in addition to the prototrophs listed in this table.

(*T−L−B_1−Lac−Het*). The possibility of further analysis was widened by the reappearance of unreduced prototrophs in the F-2 and in f-1 × p-1. The initial occurrence in the V_1^p stock of the nondisjunction-promoting factor was evidently a mutation-like accident, for retests on that stock have given no further sign of *Het*.

Considerable attention has been given to crosses of the form W-478 × W-1177 because of the wealth of segregating markers. W-1177 type stocks carrying *Lac*− and *Mal*− from which spontaneous reverse mutations (occurring at rates of the order 10^{-6} per division) can be selected by plating on medium containing lactose or maltose as the preferred carbon source have been especially useful. Dispensing with an extensive but concordant sequence of crosses carried out with less satisfactory markers, the data of Table 6 will be used to illustrate the behavior of several hundred similar heterozygotes. To isolate *Lac*+/− heterozygotes, a cross is conducted on EMS lactose agar. The 70 per cent *Lac*− prototrophs are disregarded; about ten per cent of the *Lac*+ prototrophs are unreduced for *Lac* and give *Lacv* colonies on EMB lactose. The proposed diploids are purified and maintained in lactose-synthetic medium. In some crosses, but not all, the diploid colonies can be distinguished by inspection, for they tend to grow more slowly and to have a slightly delayed fermentation reaction. A parallel procedure is used to isolate *Mtlv*, *Xylv*, etc.

The most striking feature of Table 6 is the number of entries needed. Contrary to first naive expectations, the apparent F-1 is far from homogeneous and never uniformly heterozygous. None of the cultures were *Malv* whether isolated as *Lacv* or *Mtlv*, nor has *Malv* ever been isolated from any *Het* crosses despite the most extensive trials. Most of the F-1 of W478 × W1177 were *Mal*−. But this was not the only peculiarity; of 39 *Mtlv*, 28 were *Lacv*, but 10 were *Lac*− and 1 *Lac*+. Conversely, of 28 *Lacv*, 16 were *Mtlv*, 7+, 5−.

Two interpretations for *Lac*− and *Mal*− cultures were considered. They might represent a haplogenic or hemizygous condition of the locus, or they might be homozygous "−." Reverse mutation was used as a distinguishing criterion. *Lac*+ reversions were selected on EMS lactose agar, and purified on the same medium. The reverted cultures were then streaked out on the various EMB media. Some of the reversions occurred in prototroph f-2 giving *Lac*+ no longer segregating for any marker. A total of 66 independently occurring reversions were still diploid (i.e., *Mtlv*, etc.) including selections from eight of the nine *Lac*− *Mtlv* cultures of Table 6. Without exception, each of these cultures had reverted from *Lac*− to *Lacv*. From this, it is concluded that the *Lac*− cultures are homozygous and not haplogenic. Except in consistency the behavior of *Mal*− contrasted with this. *Mal*− is much more stable than *Lac*−, limiting the number of reversions that could be selected in a moderate number of trials. In this series, seven diploid reversions from four *Mal*− *Mtlv* cultures (Table 6) were each pure *Mal*+. Similar tests on many *Mal*− diploids from a number of other *Het* crosses gave the same result. *Mal*− evidently reflects a haplogenic condition.

By inference, the *Lac*+ and *Mal*+ diploids in this series are also assumed to be homo- and hemi-zygous respectively. The only relevant evidence comes from "reverse crosses." As shown in Table 8, *B−M− Het Mal*− and *B−M− Het Lac*− were crossed with *T−L−B_1*− respectively, and the issuing heterozygous diploids collected. The transposed prevalence of *Lac*+ (together with *Lacv*) and of *Mal*+ diploids will be noted, once again supporting the concept of these mutations as inert markers. 16 *Lac*-reverted diploids from the single *Lac*−cultures of the reverse series were each *Lacv*; conversely, 11 *Mal*-reverted diploids from 7 *Mal*−cultures were all pure *Mal*+. If, as supported by the statistical results of the reversed cross, the + products of the original are equivalent to the − of the reversed, we can conclude definitely that the *Lac* locus has always appeared in diplogenic condition, whether −/−,

+/+ or +/− (*v*), whereas the *Mal* locus has always been haplogenic, whether − or +. The occurrence of homogenic factors is too consistent to allow them to be interpreted as point mutations. Furthermore, some linkage relationships, *Mal-S*, *Lac-V*$_6$, and *Mtl-Xyl* persist in the formation of the heterozygotes. The explanation we have adopted for homozygosity of *Lac* is that meiosis intervenes between the time of initial karyogamy and the establishment of the non-reduced prototroph. Such a meiosis must involve a four-strand stage to account for homozygous loci. It is thus possible that the action of *Het* is not to delay disjunction of the primary zygote but to stimulate the reunion of a pair of segregating strands. The overall process is reminiscent of somatic segregation in *Drosophila* (Stern, 1936). The possibility of concurrence of two pairs of such strands to give twin, complementary diploid prototrophs has not been realized in our experiments, except for two or three instances of twin diploids, *Mal*+ and *Mal*−, comparable to the twin haploid prototrophs discussed earlier.

The *V*$_6$−*Lac* linkage can be applied to a further corroboration of the context of this discussion. Many of the *Lac* −/− of Table 6 are *V*$_6^S$ (presumably *s*/*s*) but in some the postulated crossover has separated *Lac* and *V*$_6$ giving a *Lac* −/− *V*$_6$ *s*/*r*. *Lac*-reversions from such a culture should fall into two classes: *Lac* + *V*$_6^S$/−*r*, and + *r*/−*s*. A series of 35 independent reversions was secured. After purification on EMS lactose each reversion was allowed to segregate on EMB lactose. No more than one *Lac*− and *Lac*+ was chosen from the progeny of a single *Lacv* colony, and these were then scored for *V*$_6$. Table 7 shows how each of the 35 cultures was unmistakably either + *r*/−*s* or, + *s*/−*r*, numbering 20 and 15 respectively. (χ^2 for deviation from 1:1 is 0.7; p = .4). The result is an almost trivial consequence of homozygosity, but there are too many unexplained aspects to let any opportunity go by to test the homology of the genetic system of *E. coli* to that of other forms. Incidentally, this experiment provides another concrete instance of the familiar notion, not often directly tested, that spontaneous mutation in a homozygous cell affects the two alleles independently.

TABLE 8. NONDISJUNCTION PROTOTROPHS FROM CROSSES REVERSED WITH RESPECT TO TABLE 6.

A. *B*−*M*−*Mal*$_1$−*Het* × *T*−*L*−*B*$_1$−*Lac*$_1$−; *Lacv* selections
11 *Mal*− 41 *Mal*+ 1 *Mal*+ and *Mal*− "twin."
B. *B*−*M*−*Lac*$_1$−*Het* × *T*−*L*−*B*$_1$−*Mtl*−*Xyl*−*Mal*−; *Mtlv* selections (26 total)

No.	*Lac*	*Xyl*	*Mal*	No.	*Lac*	*Xyl*	*Mal*
12	v	v	−	1	v	+	−
6	+	v	−	1	+	+	+
2	v	v	+	1	v	v	−
1	−	+	−	1	v	+	+
1	+	+	−, + twin				

These tables should be compared with Table 6, in particular with respect to the reversal in proportion of *Mal*+:− in A., and *Lac*+:− in B., respectively.

The numerical bias in segregation from these diploids was mentioned in connection with H-1. In these experiments, it was noted that the −*r*/+*s* were readily distinguishable from the +*r*/−*s*, for *Lac*− predominated among the segregants of the latter; *Lac*+ of the former. (In other words, *V*$_6^S$ appeared more frequently than *V*$_6^r$). This type of aberration, which has been noticed repeatedly (in every diploid) may be correlated with the behavior of *Mal*.

The haplogenic fate of *Mal* and *S* means simply that these "diploids" are in fact aneuploid and it is difficult to escape the conclusion that a

TABLE 7. DEMONSTRATION OF THE TWO CLASSES OF *Lac* REVERSIONS IN *Lac*−*V*$_6^r$/*Lac*−*V*$_6^S$

Three to four *Lac*− and *Lac*+ segregants from each reverted diploid were tested for *V*$_6$, and scored as follows:

No. of diploids	Segregant Types				Class*
	Lac+*V*$_6^r$	*Lac*−*V*$_6^S$	*Lac*+*V*$_6^S$	*Lac*−*V*$_6^r$	
14	3 to 4	3 to 4	0	0	R
13	0	0	3 to 4	3 to 4	S
4	3	4	1	0	R
1	1	0	3	3	S
1	2	4	1	0	R
1	2	4	2	0	R?
1	0	1	4	1	S?

*Reversion on *V*$_6^r$ or *V*$_6^S$ chromosome, R or S. Total: 20R: 15S.

segment ("S-segment") of the genome, marked by *Mal*, *S* and a few other factors, is eliminated during some part of the sexual cycle. As a working hypothesis, we are inclined to retain a chromosomal basis for the genome, and to regard the elimination as a superimposition upon its "normal" workings. The regularity of segmental elimination and its extent makes it appear likely that at least a chromosome arm is involved, possibly an entire chromosome. It will also be noticed that, in Table 6, there is a correlation between *Mal*+ and *Xyl*+ or *Mtl*+, whereas the *Mal*− are usually either *Xyl v* or *Xyl*−. This is most readily interpreted on a crossover basis as illustrated in Figure 4.

One especially simple hypothesis for the pseudo-linkages of 58-161 × W-1177 postulated structural heterozygosity of these parents, so that, for example, a quadrivalent translocation ring developed at meiosis. This would account directly for the 4-armed "linkage map" constructed earlier, and the "diploids" (including homozygous loci) would be a result of unequal or 3 : 1 segregation of the ring such as Burnham (1948) and others have described in maize. But this hypothesis is contradicted by the results of an f-1 cross. Viable segregants from the postulated multi-armed configuration would necessarily be structurally uniform, although they might carry different genes by crossing-over. When crossed with each other, however, the segregants produced heterozygotes in which the same elimination process was repeated.

It seems likely that we have reached a point in this analysis where the genetics has overreached the essential cytogenetic framework, and possibly an *ad hoc* statement that elimination occurs is no more objectionable than speculation on specific mechanisms which may be difficult to test for the present. It may be desirable however to conclude this section with a brief generalization of the possibilities in cytogenetic terms. The occurrence of "twin" haploid or diploid prototrophs, the multi-armed linkage pattern and the regularity of *S*-elimination point to the presence of two, rather than one, chromosomes. On the other hand, some explanation is required for the aberrant segregation ratios of factors which remain heterozygous, and the *S*-elimination can be related to this most easily on a single chromosome basis. Perhaps we are relying too strongly on the perfection of specific pairing and synaptic mechanisms, for some of these peculiarities might be interpreted in terms of a high frequency of non-homologous association of

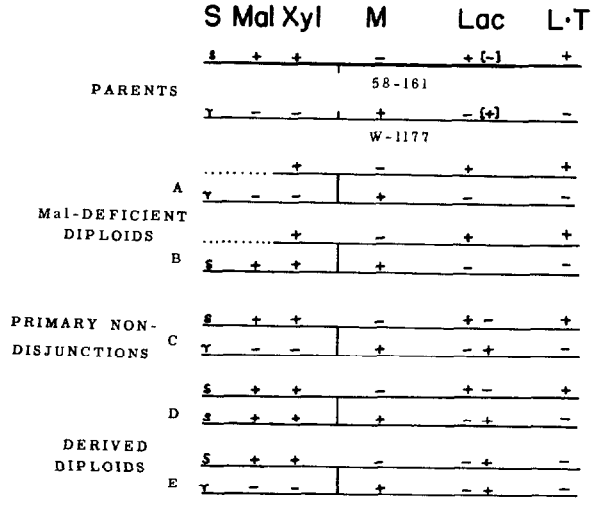


FIG. 4. Schematic constitution of diploid heterozygotes. A., B.: *Mal*-deficient diploids, as cited in Tables 6 and 7, and recovered from "Het" crosses. C.: diploids obtained as *Lac*+ non-disjunctions from crosses of Lac_1- × Lac_4-. D., E.: spontaneous changes from C. E. is auxotrophic and suitable for use in diploid × haploid crosses.

the sort invoked by Longley (1945) and Rhoades (1942) to explain false linkage and non-random segregation in maize. As far as we know, however, there is no precedent for dissynchronous reduction of the kind which might be postulated to explain twin prototrophs.

The discussion to this point has been confined to the unreduced progeny of crosses involving *Het* which came from the unique accident of H-1. Once such nondisjunctional types were realized, the possibility of their more general occurrence was reviewed. A few thousand prototrophs had previously been streaked out on EMB lactose agar, and we were fairly confident that the *Lacv* character would not have been overlooked. A more effective screening technique was therefore devised to look for the occurrence of non-reduced prototrophs by the recurrence of *Het*, environmental effects, or other mechanism. Previously, a lactose-negative mutation at a new locus had been isolated (W-67, *B*−*M*−*Lac*−) and distinguished from Lac_1- (as carried by W-1177) by the symbol Lac_4-. Crosses of Lac_4- with Lac_1- resulted in prototrophs, about 0.1 − .3 per cent of which were lactose-positive, so these factors are very closely linked, but separable. It was anticipated that *Lac*+ factors generally would be dominant. Consequently, recurrent heterozygotes should be detected more efficiently by testing only the lactose-positive prototrophs in a cross Lac_1- × Lac_4- (e.g., W-67 × W-1177). The rare

lactose-positives can be detected by simple inspection of prototrophs on the original crossing plates of EMS lactose agar. Unfortunately, W-67 is relatively infertile, but adequate yields can be obtained. These anticipations were soon rewarded, for about a third of the lactose-positive prototrophs from such crosses were *Lacv*. Thus, standard stocks produce nondisjunctional progeny but some hundred times less frequently than *Het*. These progeny probably are not a result of new *Het* mutations, judging from f-1 tests, but *Het* itself has not always been transmitted to the f-2 in comparable crosses. About 90 per cent of these "primary" nondisjunctions have behaved like the *Het* F-1 and F-2, particularly in exhibiting hemizygosity for *Mal*−. Of course, since their constitution is +−/−+ with respect to Lac_1 and Lac_4, the segregants are all lactose-negative, barring a negligible frequency of crossing-over. Thus the colonies on EMB lactose tend to a periclinal rather than a sectorial type of variegation. For certain studies, it is very useful to be able to use the *Lac*+ and *Lac*− appearance as criteria of the diploid or haploid condition of a given colony.

In four or five cases the primary nondisjunctions have been *Malv* exceptions to the rule of *Mal* haplogenicity. These stocks have also shown biased segregations, but the possibility of aneuploidy in other segments has not been ruled out. In these diploids, *Mal*+ is dominant to *Mal*−, and their existence itself shows that the nonoccurrence of *Malv* in the *Het* progenies is no artefact. Table 9 shows segregation patterns from both types of diploid. Lac_1− unfortunately is not easily distinguishable from Lac_4−, and no attempt to map this with the other factors has so far been made.

That segregation is always concordant among the *Het* diploids has already been mentioned. Extensive searches for "partial" segregants e.g., *Lacv Xyl*−, or auxotrophic *Lacv* from *Lacv Xylv*, etc., were unsuccessful, although one or two exceptions occurred in old cultures under circumstances that did not rule out a repeated sexual generation. The primary *Malv* nondisjunctions behave somewhat differently: one or two per cent of the colonies that had apparently segregated for *Mal* (pure maltose-negative) remain *Lacv*, and conversely. Thus secondary *Lacv Mal*−, and *Lac*− *Malv* cultures are produced. If S^r/S^s is segregating (H-267) the former are readily detected as *Lac v* colonies on streptomycin medium (which inhibits both the original *s/r* type, and S^s segregants). One might anticipate that these secondary types would resemble the previous *Lacv Mal*− cultures, and that the partial segregation resulted from segmental elimination such as occurs in the formation of *Het* diploids. However, all of these secondary *Mal*− cultures tested by reversion for *Mal* have consistently yielded *Malv*, not *Mal*+. The aspect of *Het* behavior dup-

TABLE 9. SEGREGANTS FROM HETEROZYGOUS DIPLOIDS

	H-212 ("Het" diploid: See Figure 4A)			
Proportion calculated from combined data*	*Lac*+*Xyl*+*Mtl*+	*Lac*−*Xyl*−*Mtl*−	*Lac*−*Xyl*+*Mtl*+	*Lac*−*Xyl*−*Mtl*+
	.18	.61	.15	.05

	H-226† (Primary Non-Disjunction: See Figure 4C)			
	Mal+*Xyl*+*Mtl*+	*Mal*−*Xyl*−*Mtl*−	*Mal*+*Xyl*+*Mtl*+	*Mal*+*Xyl*−*Mtl*−
Numbers from:				
A. "Random" *Lac*−, isolates	86	4	1	0
B. *Mal*+ and −fixed	52	57	5	3
C. Clones in cell pedigrees	7	10		

*Several hundred variegated colonies on EMB lactose or xylose agar were streaked out, and a pure + and a pure − isolated from each one. To rectify the bias imposed by thus fixing the ratio of + to −, data from the two sugars are combined for the computation of the unbiassed frequencies. This does not compensate for perturbations due to selective differentials of other loci.

†All segregants from this balanced heterozygote are *Lac*− (Lac_1− or Lac_4−), so that may be isolated "randomly" as single *Lac*− from individual *Lacv* colonies. The unbalanced ratios under A. are probably a result of differential growth. More precise clonal data, as in C., (Zelle and Lederberg, 1951, and unpublished) cannot be collected on an adequate scale, but do suggest that the "random" statistics are biassed. The low frequency of crossing-over is characteristic of this group of diploid cultures.

licated in the secondary types is thus the occurrence of homozygous loci rather than elimination. The distinctive behavior of the secondary *Mal*– cultures reinforces the previous test for hemizygosity. The linkage between *Mal* and *Xyl* in the formation and segregation of primary nondisjunctions was noted earlier. However, one *Mal* –/– *Xyl* +/– allowed a test of the random occurrence of *Mal* reversions similar to that of Table 7 for *Lac* –/– V_6 *r/s*, and with the same result (11 *Mal Xyl* +–/–+; 17 ++/––., p = 0.25). The derived *Lac* – *Malv* partial segregants are homozygous *Lac*– by a similar test.

An especially interesting class of partial segregants is that in which auxotroph factors have become pure—(presumably homozygous) while *Lac* or *Mal* or both remain heterozygous. Such auxotrophic diploids can be crossed readily with complementary auxotrophs (haploid or diploid), to give large yields of F-2 heterozygotes. Studies in progress indicate that segmental elimination does *not* occur during these crosses. However, crosses of f-1 from primary nondisjunctions show the same trend of S-elimination as the other f-1 tested. These 2n × n and 2n × 2n crosses are expected to be especially useful in further work for three reasons: the non-occurrence of S-elimination, the high proportion of heterozygous progeny, and (in principle) that chromosome recombination rather than specific crossovers is the necessary condition for the formation of detectable prototrophs. Since the yield of prototrophs from such crosses is perhaps tenfold that of haploid crosses, we may reinforce our earlier contention that a fair proportion of zygotes produce prototrophs. The S-elimination was especially troublesome for the problems of constructing heterozygotes for dominance tests of the genes involved (e.g., S^r/S^s), and a good deal of fruitless effort could have been avoided.

Considerable space has been devoted to the present involved status of the formal genetics of *E. coli* K-12, not because of its intrinsic importance, but because it is a necessary foundation for the evaluation of the application of recombination to genetic problems. I think everyone, including the protagonists, would agree that much of the controversy in the genetics of yeast devolves upon very similar questions on the distribution of chromosomes at meiosis. The foundations of yeast genetics are older and broader than those of *E. coli*, and the advantages of a morphologically well-understood life cycle, the coherence of meiotic products in the ascus, and matings under the control of heterothallism, are not yet shared by the bacterial geneticist. This contrast provides all the more reason for attempting to build a detailed, incontrovertible foundation for theoretical construction in this field.

It may be pertinent to enquire at this point whether the entire approach to the analysis of *E. coli* segregations in chromosomal or cytogenetic terms may not be fallacious—whether there may not be an entirely unique genetic mechanism involved. To recapitulate, at the risk of redundancy, the primary nondisjunctions are the clearest examples of bacterial hybridity. They are unstable in respect to those genetic factors which delineated the parental bacteria, and in no others. The association of these factors is intracellular, as proven by direct single cell isolations. The factors from the individual parents show a strong tendency to separate in the same blocks or combinations, but the linkage is not absolute and all categories of recombinations occur. Every heritable character for which a mutation has occurred to permit a test is included in this segregation system. The evidence for a uni-linear association of factors is quite incomplete, but some groups of factors are most likely organized in this way (e.g., V_6 – *Lac* – V_1 from haploid data). Although the detailed organization of the genetic structures is still obscure, their resemblance to chromosomal systems is incontrovertible: linkage; high frequency of 2n output in 2n × 1n crosses; coupling and repulsion phases —by mutation in the diploid; response to mutagenic agents; dominance and over-dominance (*vide infra*); complementary segregation (qualitatively); and permutation of segregation ratios in reverse crosses. The anomalies which must be explained include the regular elimination in most crosses of a particular segment of the genome, segregation ratios deviating from the expected 1 : 1, and difficulties in a comprehensive linkage analysis. At first sight, an appeal to chromosomal anomalies may appear to be too special to be justifiable, but we have found no alternative approach which provides a more fruitful working hypothesis. It should be kept in mind that *Drosophila* and maize are popular for genetic work precisely because of the usual regularity of their segregation behavior, and that there are whole orders of arthropods and many plant species (as M. J. D. White, C. W. Metz or R. E. Cleland would testify) whose genetic behavior would seem to show much more profound inconsistencies with a chromosomal interpretation than does that of *Escherichia coli* K-12. At any rate, we now have good "reason to believe that the bacterial

cell contains a special genetic substance or structure, differentiated to perform genetic functions..." (Taylor, 1949).

Correlating Cytological and Genetic Investigations

Other speakers at this symposium will have discussed in more detail the present status of bacterial cytology and its bearings on bacterial genetics. A number of workers have presented convincing evidence for the presence of nuclei in bacterial cells, but their identification as nuclei has hitherto been based only on incomplete morphological and cytochemical evidence, in the absence of any more direct opportunity to locate the genes within them. A most attractive objective would be a documentation of the nuclear events associated with genetic recombination in *E. coli* K-12, or any other suitable organism, but this is on the horizon, not at hand.

Meanwhile, many investigations of mutagenesis have been predicated on probably fallacious models of bacterial cells as constructively isolated genes, despite the contrary cytological evidence for the multi-nucleate condition of most rod-shaped bacteria. Many of the characters used in bacterial mutation research are recessive (e.g., resistance to phage or streptomycin) so that mutations induced in multinucleate cells could not begin to exert their phenotypic effect until nuclear separation has occurred. In this respect a comparison of vegetative cells with presumably uninucleate endospores might be fruitful.

The establishment of nondisjunctional or "diploid" cultures opened the question of a cytological comparison of 2n and n for the purposes of a bacterial cytogenetics. For some time, preparations like that illustrated in Figure 5, have encouraged this hope. Diploids often show cells of greater uniform length than haploids, and with chromatinic structures of greater apparent complexity. Very often, there appeared to be two larger, more dispersed "nuclei" per cell, in contrast to two pairs of more condensed nuclei that often characterize comparable haploid cultures. The structure of the "nuclei" is obscure, for we had been unable to analyse the connections of the granules to determine whether they form a single connected group or several groups. So far, our material, interpretations, or techniques (HCl-Giemsa) have not sufficed to demonstrate clear mitotic figures, but there are many unmistakable examples of symmetrically placed groups of chromatin both in haploid and diploid cells. The preparations so far studied do not admit of any clear interpretation in terms of doubled chromosomes, and it is not yet excluded that the differences reside principally in a better expansion and resolution of nuclear structure in the diploid cells. In occasional preparations haploid cultures have shown nearly the same order of complexity in chromatinic structure as diploid (Figure 6), but to date one of us has consistently been able correctly to classify stained smears prepared by another, ostensibly by virtue of the nuclear cytology. On two occasions, a cytological determination correctly anticipated a later genetic definition of the status of a culture (one was a secondary *Lac*+ homozygote; one a haploid culture carrying an unstable gene which simulated the variegation of heterozygosity). The further cytological analysis may well rest upon technical and conceptual advances of the kind discussed elsewhere in this symposium.

Stempen (1950) and others have reported that nuclei can be visualized in living bacteria by phase contrast microscopy. This technique has remarkable advantages for observing cells as a whole, but only faint suggestions of the nuclei are apparent in preparations of *E. coli* K-12. There is considerable fluctuation in the definition of the presumed chromatin (which appears light

Plate I

FIG. 5. Haploid parent, W-67 (a), and diploid offspring, H-226 (b). Giemsa stain following osmic fixation and hydrolysis with HCl.

FIG. 6. Haploid cells, K-12. Giemsa-osmic-HCl.

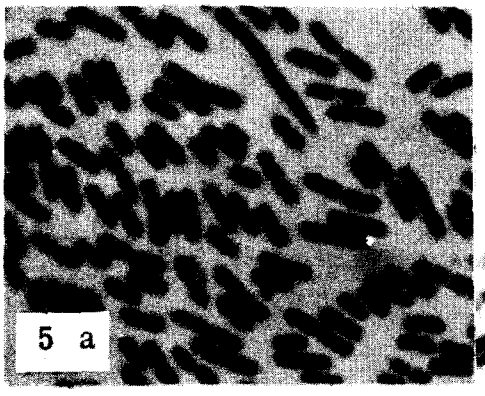
FIG. 7. Genetic effects of ultra-violet light on a diploid culture, H-226. a. Control plating showing preponderance of balanced lactose-positive colonies (Lac_1^-/Lac_4^-; see Figure 4C) on EMB lactose agar. b. Comparable plating of an aliquot exposed to ultra-violet light.

FIG. 8. Phase contrast photomicrographs of microcolonies. a. Control plating of strain K-12. b. From diploid cells, H-267, exposed to ultra-violet light.

FIG. 9. Cytological effects of ultra-violet light on a diploid culture, H-267. a. Microcolony from control suspension. b. Microcolony from treated suspension. Giemsa-osmic-HCl.

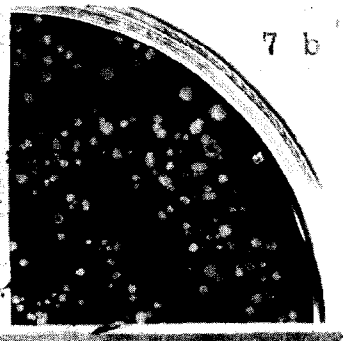
FIG. 10. Mutability differences between Lac_1^- alleles. a. Mutable, Y-87. b. Stable, W-112. Both on EMB lactose agar, 48-hour plates.

FIG. 11. "Large Bodies" from *Salmonella* filtrates, exposed to antiserum. The bacteria were artificially added to provide a size standard.


5 a


5 b


7 a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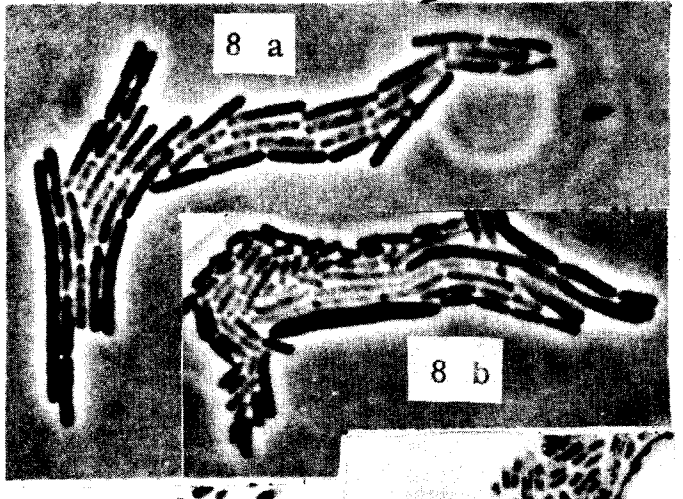
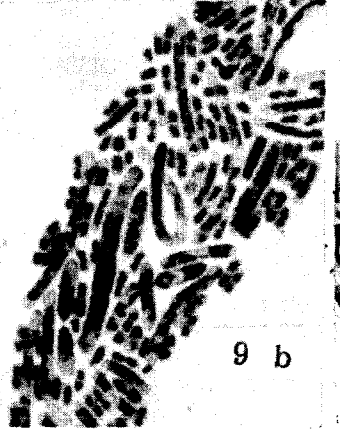
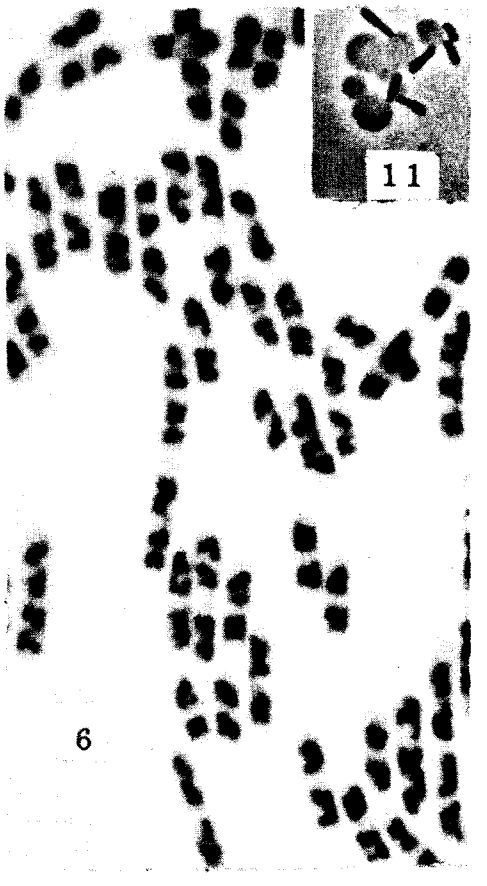

8 a

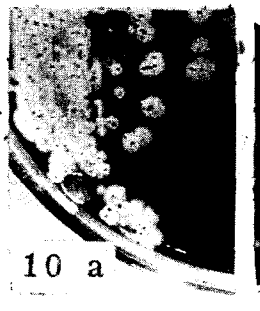
8 b

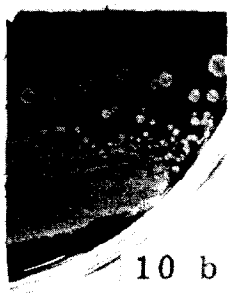

9 a


9 b


6

11


10 a


10 b

in the photographs, Figure 8). We have not yet succeeded in identifying 1n *versus* 2n cells by this method, except by the uncertain criteria of size and growth rate.

One question that is intimately concerned with nuclear behavior is the mechanism of bactericide, especially by mutagenic radiations and chemicals. The first order kinetics of sterilization often observed with disinfectants has led to the suggestion that lethal mutations of some sort might be implicated. Haploid cells offer no opportunity to study the possible genetic mechanisms of their death, but diploid cells should allow of the recovery of the presumed lethals in heterozygous condition.

Because of the readiness with which the diploid state is detected, *Lacv Malv* primary nondisjunctional types have been used in this analysis. Owing to their Lac_1 Lac_4 +−/−+ constitution, all the haploid segregants are lactose-negative, in contrast to lactose-positive diploids. It was soon found that the UV-sterilization responses of the diploid (H-226) and the haploid K-12 were congruent, both showing a target multiplicity of several hundred.

The behavior of diploid cells surviving UV is illustrated in Figures 7 and 9 from a genetic and cytological viewpoint, respectively. At first glance, it appeared that an early effect of UV was to induce haploidization, 50 per cent or more of the colonies showing this effect with a survivorship of 90 per cent, so that selective survival is disqualified. This would make for a simple picture if the genome, chromosome, or haploid nucleus were the unit of inactivation, haploidization would be a halfway step to "nulliploidy" or death. This view became difficult, however, when it was found that increasing doses of UV did not continue to increase the proportion of apparent "haploid" survivors whose frequency stabilized at about 80 per cent of the total survivors. It became untenable when the plates were incubated for longer periods and inspected more closely. In the center of nearly every "haploidized" colony, there developed a lactose-positive spot from which a diploid *Lacv* could be isolated. The surviving cell evidently had retained the potentiality of transmitting the diploid condition. However, if weakly irradiated cells are incubated for a few hours in broth virtually all of the colonies then formed on agar are truly haploid. The surviving cell is so altered that haploid cells are segregated and grow at a normal rate, but a residuum remains which recovers only later to form a diploid cell growing normally. This is shown even more clearly by the following experiment:

A drop containing about 10 cells from a diploid culture is streaked along a line at one edge of a plate. Some of the plates are irradiated. After a few hours incubation, a glass spreading rod is firmly stroked once, perpendicular to the line. This disperses the cells of individual microcolonies in lines across the plate. Most of the progeny of unirradiated cells remain typically diploid. Many haploids are segregated during the first few hours from irradiated cells, leaving other cells producing diploid colonies whose appearance ranges from the typical to the central spot type.

The likely cytological correlate of this behavior is found in the "snakes" or long filamentous bacteria observed by many authors after UV treatment. The cell later forming a "snake" may first divide to form a clone of rapidly dividing smaller cells (haploid?) in which the "snake" remains relatively dormant for long periods (diploid residuum?). The chromatinic material of some of these cells is highly disorganized, aggregated, and pycnotic, while the total nuclear material corresponds to that of dozens of ordinary cells closely packed in one filament. It is not clear why haploid segregants should recover preferentially, but it is certain that there is every opportunity for all varieties of genetic dis- and re-organization. This is reflected in the rather high proportion of lactose-positive crossovers (Lac_1 + Lac_4+) found among the segregants, and especially in the very high frequency (50% or more) of partial segregants among residual diploids.

Clark *et al.* (1950) have reported that UV stimulates prototroph formation. If the effect is not an artefact due to increased residual growth in the minimal agar, it may conceivably be related to the centripetal condensation of nuclear material in irradiated cells, but only if cytogamy normally is more frequent than karyogamy. Their discussion, however, includes the speculation that the irradiation "could cause increased genetic transfer of genetic material between the two strains... by recombination by the organisms of desirable genetic properties in an attempt to overcome undesirable side reactions that result from the treatment"—a point of view that we cannot share.

The study of UV effects was initially undertaken with the expectation that diploid cells

would be more resistant than haploid, and that recessive lethals would be detected in diploid survivors by their effect of inhibiting effective segregation. The auxotrophic mutations, which can be regarded as "lethal" on synthetic medium, provide a clear model for this behavior, for segregation is very much reduced on this medium owing to the growth requirements of the auxotrophic segregants. However, no clear cases of balanced lethal survivors have been found, and lethals presumably play a very limited role in bactericide. The development of clones from irradiated cells does not, however, follow an uncomplicated course, and it is possible that lethal-carrying genomes are selectively eliminated to some extent. In an earlier, similar study, Atwood (1950a, b) found lethals, probably deletions, in *Neurospora* heterokaryons, but not in sufficient numbers to account for UV sterilization of conidia.

At first sight, these results may appear to conflict with reports that UV and X-ray sensitivity in yeasts are inversely correlated with their ploidy. Atwood and Norman (1949) found a similar correlation in multinucleate *Neurospora* conidia. The correspondence between the ploidy or number of nuclei and the radiobiological "target multiplicity" has been taken by certain authors as evidence for recessive lethals as the specific genetic mechanism of killing. This is, of course, fallacious: the target multiplicity based on lethals would be larger than the ploidy by a large factor depending on the total number of loci.

When a radiation resistant mutant of *E. coli* B, B/r, was first reported by Witkin (1947), the suggestion was considered that the resistance might be accounted for by genetic reduplication. It was carried further by Buzzati-Traverso, *et al.*, (1948), who suggested tentatively that certain strains of *E. coli* might be polyploid on the basis of a correlation between apparent nuclear volume and radiation resistance. At least for B/r, the suggestion should have been settled by the frequency of occurrence of recessive mutations, (Demerec and Latarjet, 1946). It is now apparent that a great many environmental variables can influence bactericidal effects of radiation, and we may presume that some of these can be imitated by genetypic variation. At best, radiobiological kinetics cannot be relied upon as the principal basis of any genetic interpretations.

A mechanism of bactericide by UV not yet considered here is the activation of latent bacteriophage, as described by Lwoff, *et al.*, (1950).

TABLE 10. EFFECTS OF BACTERICIDAL AGENTS ON DIPLOID *Escherichia coli*

Radiomimetic	Non-Radiomimetic
X-ray	High temperature
Ultra-violet	Streptomycin
N-Mustard	Methyl green
Formaldehyde	Urethane
Hydrogen peroxide	Ninhydrin
Acetic anhydride	Iodine
Acetyl chloride	Iodoacetamide
Dimethyl sulfate	Sodium desoxycholate*
Ethylene oxide	Acriflavine*

*Inconclusive owing to severe clumping or inadequate bactericidal effect.

A similar process occurs in lysogenic *E. coli* K-12, and probably accounts for an appreciable part of UV bactericide under some conditions. The general cytogenetic features of UV-response mentioned previously for lysogenic diploids hold for the irradiation of non-lysogenic cultures as well, but a detailed analysis has yet to be carried out to determine the role of this effect under the conditions of our experiments.

Insofar as more than 50 per cent of the cells exposed to UV may show genetic responses to very small doses having little bactericidal action, the diploid cell constitutes a very sensitive system with which to test other agencies for "radiomimetic" effects. Preliminary studies have been initiated with X-rays. At the lowest dose tested, 1000 r, a survival of about 90 per cent was observed, and already at least a fourth of the survivors showed the type of colony illustrated in Figure 7b for UV action. With 10,000 r, survival was 30 per cent, and 81 per cent of the survivors were "haploidized." But increasing doses did not increase this proportion, possibly because the criterion for the radiomimetic effect is not absolute, and varies with the time the plates are incubated. A more suitable procedure would count the proportion of pure haploid colonies appearing after a brief time of incubation in broth. This would undoubtedly greatly amplify the apparent effect, but might obscure its complexity. As far as we know, this is the most sensitive radiobiological response observed with microorganisms, for which the usual doses are measured in tens and hundreds of kiloroentgens.

Although the genetic or physiological basis of death is still not well understood, the induced haploidization can be used to classify chemical bactericides as radiomimetic or otherwise, along the lines of cytopathological studies with plant

and animal cells. As might be expected, the effects of UV, X-rays, and nitrogen mustard have been indistinguishable in this system, except that those of UV are reversed by visible light in parallel with the photoreactivation of viability. A group of other chemicals have been tested also. As some of these are quite unstable in aqueous solution, the tests have been carried out with five and ten minute exposures, usually in M/10 buffer with or without added calcium carbonate. The cells are washed in the buffer, and the system is diluted (usually to 10^{-5} or 10^{-6}) to terminate the exposure. Table 10 summarizes the tests of these compounds for radiomimetic effects.

It will be observed that all of the active compounds, except H_2O_2 are effective reagents for substituting labile organic H– with alkyl, acyl, or similar groups. It is quite possible that hydrogen peroxide may act similarly via the formation of reactive alkyl peroxides. It has been suggested that UV may act indirectly via the formation of hydrogen peroxide in aqueous medium, but the effect of hydrogen peroxide differs from UV in its insusceptibility to photoreactivation.

Most of the compounds listed here as radiomimetic have been reported by other workers to be effective mutagens. But the chemically nearly inert mutagens, caffeine and urethane, are not radiomimetic, and may be presumed to have a different mode of action. Whatever the intimate mechanisms of action, it appears likely that many disinfectants act in part via effects on the genetic mechanisms. However, other agents including streptomycin, heat, iodine, and phenol show no such effects in this system. If a model of untreated bacteria as essentially equivalent to suspensions of isolated genes for mutation study is oversimplified, a similar consideration of irradiated bacteria is completely fallacious. Owing to the multinucleate condition of the starting material, *E. coli* is not entirely suitable for detailed cytogenetic analysis of radiation effects, but the provocative question may once again be raised whether the genetically localized effects of mutagens may not be to some extent secondary concomitants of recovery from what have too often been dismissed as the "physiological" effects on nuclei and chromosomes, rather than reactions of individual gene molecules.

PHENOGENETICS

The most critical application of recombination analysis concerns the identity of mutations as alleles. This question arises frequently in two connections: in comparing different deviations from wild type (heterotypic mimics—such as different lactose-negative or drug-resistant mutants), and in testing apparent back mutations to determine whether the phenotypic reversal is due to reverse or to homotypic mimic mutation. The methodology of the two tests is very close. For the first, the different mutants are crossed with each other, and the progeny examined for recurrence of the wild type (Ab × aB = AB, but $a \times a^1 \neq A$); for the second the reversal stock is crossed to type, and the recurrence of the mutant is looked for: (aM × Am = am, but $A' \times A \neq a$). If, as is often the case, it is possible to devise a selective or at least an indicator medium to detect the decisive nonparental genotypes by inspection, a very large number of tests may be conducted with reasonably small effort. For example, in intercrossing *Lac*– mutants, 400–500 prototroph colonies on a plate can be scored at a glance for the presence of *Lac*+, so that a single moderate experiment of 40–50 plates will constitute 20,000 tests for crossing-over. In contrast to similar, albeit more laborious, studies in maize and *Drosophila* (Laughnan, 1949; Green and Green, 1949), the chief limitation is the frequency of spontaneous "mutations" rather than the collection and classification of so many offspring. Crossing-over has been used primarily for the analysis of the grosser structure of the genome and a new type of relationship, pseudoallelism, is being found at the very margin of technical possibilities in higher organisms. The closer relationships of genetic units, of associations which may be represented by linkages of .01 and .001 *centimorgans*, are an important challenge to the versatility and technical plasticity of microbial genetics.

The first application of recombination to the differentiation of phenotypically similar mutations was the separation of two main types of resistance to the virus T1. Demerec and Fano (1945) found, in *E. coli* B, the types B/1 and B/1,5(= B/5,1) which were resistant to T1 only, or to both T1 and T5, respectively. They suggested that these were genotypically distinct effects, but pointed out, rightly that a proper genetic test required a sexual phase to allow crosses, then unknown. Similar phenotypes have been detected in *E. coli* K-12 and fortuitously named V_1^r and V_{1a}^r (for "/1,5" and "/1" respectively). Intercrosses of a number of V_1^r stocks have given only this type; similarly for V_{1a}^r; but $B-M-V_{1a}^r \times T-L-V_1^r$ gave about 15 per cent T1-sensitive (V_1^s V_{1a}^s) recombinants. This was the first example of a recombinant which was

phenotypically more than a reassortment of the overt parental *characters*. Several authors (see Luria, 1946) have also commented on the (infrequent) occurrence in *E. coli* B, of complex resistance patterns in a single selective step, which look superficially like the superposition of simple patterns (e.g., B/1,5, 3,4,7), and of associations between auxotrophy and resistance. Efforts to find a genetic rationale for complex resistance have been blocked by our failure to demonstrate such types in *E. coli* K-12.

The genetics of another T1-(partially)-resistant mutant, V_1^p, was mentioned in the account of *Het* nondisjunctions. No T1-sensitive crossovers were found in several hundred additional tests of $V_1^p \times V_1^r$ but the compound V_1^p/V_1^r is fully sensitive to T1 and T5. We are evidently dealing with pseudo-alleles of the type discussed by Stephens (1948), wherein a relatively limited test fails to show crossing-over between units whose physiological differentiation is clearly shown by their interaction in heterozygotes. It would be interesting to compare recurrences of V_1^r of spontaneous and induced origin for their interaction with V_1^p. Unfortunately, V_1^s cannot be scored except by tests on individual colonies, and is therefore not suitable for large scale recombination tests.

Another example of heterotypic mimics is the set of lactose-negative mutations. These are readily detected as light-colored sectors or colonies on EMB-lactose agar, and occur with a frequency of 1 to 5×10^{-4} in heavily irradiated populations. About 300 independent occurrences of *Lac*− mutations were isolated from the inspection of approximately a million colonies during 1948–1949. This program was initiated as a specific test of the "one-gene—one-enzyme" hypothesis which at one time suggested that each enzyme was a specific product of a single gene, and that individual genes probably functioned *via* the activities of a single enzyme. This hypothesis had been supported by previous work by Beadle, Tatum, Horowitz, Bonner and others on *Neurospora* to the effect that most of the analysable auxotrophic mutants involved specific chemical blocks, including some which at first sight appeared to be more complex (e.g., the isoleucine-valineless). Horowitz has gone to great pains to reply to the criticism voiced here by Delbrück five years ago that many pleiotropic mutations might be lost if one of the effects were irreparable. It may be questioned whether this question goes to the heart of the matter, although it will doubtless arouse a good deal of discussion. What we should like to see as evidence for the theory is a two-fold demonstration: a) that all of the mutations directly affecting a single, well defined step are isolocal, and b) that the effects of all mutations at this locus are confined to this step. Aside from the purely technological problem of accumulating the necessary numbers of recurrent mutations in *Neurospora*, it is doubtful whether the first criterion could really be satisfied with present methods. The modalities of enzyme formation and action are so obscure that exceptions to monomorphic action are readily assimilated within our ignorance. But perhaps more crucial is the difficulty of formulating a precise defini-

TABLE 11. LACTOSE-NEGATIVE MUTANTS OF *Escherichia coli* K-12

Locus	Phenotypic effects	Other remarks
Lac_1	5–10% residual lactase; responds optimally to alkyl galactosides.	Frequent recurrences to alleles of varying revertibility; may be a complex locus.
Lac_2	Very little or no residual lactase.	Single occurrence in present series.
Lac_3	Glucose-, maltose-negative.	Several occurrences with same pleiotropic effect.
Lac_4	No residual activity.	Frequent recurrences. Very closely linked to Lac_1.
Lac_5	Ferments maltose, gluconate poorly.	Several recurrences with same pleiotropic effect.
Lac_6	Ferments galactose slowly.	
Lac_7	Very little or no residual lactase.	
Others	Ferment hexoses or all carbohydrates poorly; probably affect intermediary metabolism.	

tion for a single gene, as other speakers at this symposium will have pointed out.

The utility of lactose fermentation for such studies ensues from the ease with which mutants can be obtained and intercrossed for genetic purposes, and from the technical facility of assay and characterization of the enzyme (lactase or β-galactosidase, Lederberg, 1950c; Cohn and Monod, 1951) that mediates the key step. Most of these 300 mutants have been intercrossed, (not in all possible combinations, but with the established type stocks), and the results call for a group of at least seven loci, such that crosses of isolocal mutants give only *Lac*− prototrophs; whereas a heterolocal cross gives a proportion of *Lac*+, as summarized in Table 11.

The only difficulties in genetic differentiation have involved Lac_1− and Lac_4−. These are so closely linked that several thousand prototrophs must be examined to find *Lac*+ with assurance; some of these are nondisjunctional heterozygotes, but crossovers are also observed.

The Lac_1− series has been selected for further investigation because of the richness of the available material. A special study has been made of the reverse-mutation potentialities of different Lac_1− recurrences (E. Lederberg, 1948; 1950). Some cultures have never been observed to revert, ($Lac_1{}^{-st}$); others, ($Lac_1{}^{-m}$) show numerous reverted papillae in every colony on EMB lactose agar, (Figure 10), and intermediate rates have also been noted. The reversions have been tested extensively in crosses to *Lac*+, and found to be bonafide reverse-mutations both phenotypically and genetically. The reversions have always shown the same stability as the original *Lac*+. It should be pointed out that even the so-called mutable alleles have mutation rates of the order of 10^{-6} or 10^{-5} but selection rather than instability is responsible for their proclivity to revert on lactose agar. Inherited shifts to a lower grade of mutability have been found both in untreated cultures, and especially following UV treatment. Despite extensive observations, however, no heritable upgrades were noticed. Papillating colonies are a favorable system for genetic studies on mutability as such, for each colony on a plate provides a rough but direct score of this attribute. Without putting undue emphasis on the superficial resemblance between Lac^{-m} and *dotted* corn, we looked for mutations that might correspond to *Dt-dt*. Many shifts to lower mutability proved to be interallelic, i.e., crosses of the derived Lac^{-st} with *Lac*+ gave only the parental types. Some of them, however, are the result of mutations at other loci, for crosses with wild type gave Lac^{-m} of the original grade, as well as the parental Lac^{-st} and *Lac*+. Further consideration of the mutations affecting the Lac^{-m} phenotype has, however, failed to provide convincing evidence of a bonafide *dotted*-like effect on mutability *per se*. The interactions are probably phenotypic.

Once such interaction was predicted *a priori*: if a second Lac_x− mutation occurred in the $Lac_1{}^{-m}$, it would effect a stabilization of the *Lac*− phenotype. Reversions at Lac_1 would leave the cell Lac_x− and *vice versa*, leaving very little opportunity for phenotypic reversal. This mechanism for stabilizing heterotypic phenotypes has been postulated as playing a role in evolutionary specialization and exemplified as a laboratory curiosity in *Neurospora*, (Mitchell and Mitchell, 1950). In *E. coli* it would be experimentally rather difficult to distinguish this from a *dotted*-like effect. $Lac_1{}^{-m}$ $Lac_7{}^{-m}$ obtained by recombination were Lac^{-st} as expected. In one instance, the suppression of mutability was associated with a loss of the ability to ferment butyl galactoside. This is characteristic of other *Lac*− mutants, and the phenotypic effect itself contrasts the mutation from *dotted*. In most instances, the second, mutability-suppressing (*ms*) mutation had an obvious effect on the expression of *Lac*+ that facilitated the identification and characterization of *ms*. Some *ms* simply inhibited glycolysis, (e.g., one owing to a nutritional deficiency for adenine plus thiamine), and directly reduced the selective advantage of *Lac*+ needed for the expression of reversions as papillae. Others had more unexpected effects: one *ms* was identified as a *Gal*− (galactose) mutation which appears to have a dual effect. As compared with a *Gal*+ background, the *Lac*+ is slightly less effective. Probably more important, the residual galactosidase activity of the *Lac*− is increased, (though not enough to allow *Gal*− to be classified as a suppressor of homotypical mimic), so that the selective differential between *Lac*− and *Lac*+ mutations is diminished. The effect of the *Gal*− mutation in enhancing the type function of $Lac_1{}^{-m}$ is no more perplexing than the fact that butyl galactoside evokes lactase from this mutant, whereas lactose does not. (The residual lactase activity is about 10% of normal at full enzymatic adaptation). This separation of the specificity of the enzyme-forming mechanism from that of the enzyme itself leads to the paradox that lactose-grown cells are much less well adapted to lactose than cells grown on butyl galactoside.

Homotypical mimics of *Lac*+ have been detected in cultures of Lac_1–st incubated in lactose medium for several days. Their occurrence in Lac_1–^{m} is presumably overshadowed by the more prominent and earlier developing *Lac*+. Several loci appear to be involved, but their relationships to other "*Lac*" loci are not known. None of these mimics equals the original or revert *Lac*+ in intensity of fermentation—possibly a question of the adaptation of a single gene to the genetic background. This may be compared with the incomplete "reversions" of auxotroph mutants reported by Davis (1950a). These have not been studied genetically to assure that they are not mimic mutations.

The concept of allelism has been verified objectively in the following ways: a) phenotypically diverse mutations such as Lac_1– and Lac_3– have always recombined; b) allelic identities have been consistent and unambiguous, except for the closely linked Lac_1, Lac_{1a}, and Lac_4; c) crosses of a Lac_1– mutation (53m) with the same Lac_1– allele (extracted from a heterozygote with *Lac*+) gave no *Lac*+ in over 20,000 tests. For these reasons, we are convinced that these results can be profitably compared with similar work on *Drosophila*, *Neurospora* and *Zea*. This conclusion is emphasized because some of our crossingover experiments point to some complexity of genic structure. The following discussion revolves around three occurrences of *Lac*–: Lac_1–^{87m}; Lac_1–^{53m} and Lac_1–^{112st}. At their initial occurrence, the first could be crossed to the other two, and the results of a few thousand tests pointed to their iso-local identity. One or two *Lac*+ prototrophs were noted, but were at first ascribed to the mutability of the *m87* allele. Closer scrutiny showed, however, that *Lac*+ was a consistent occurrence in crosses of 87m × 112st (6/10,000), but not in 87m × 53m (0/60,000), thus reopening the question. However, since the EMS lactose crossing medium might select for infrequent *Lac*+ reversions occurring in *Lac*–^{m} prototrophs, a further check on this possibility was desirable. This was afforded by a linkage test similar to that used by Laughnan (1949) in the dissection of the *A* locus in maize. An m87 V_6^r stock was prepared and crossed with *st-112*. Owing to the linkage of V_6 and Lac_1, most of the *Lac*+ arising from the mutable *Lac*– should remain V_6^r. On the other hand, if the sequence of factors were (*st-112; m-87;* – V_6), most of the crossover *Lac*+ would be V_6^s. The latter was the case in 11 out of 14 *Lac*+ prototrophs isolated. The *st-112* locus was therefore distinguished as Lac_{1a}–.

The question then arose: how would Lac_{1a}– behave in combination with Lac_1– in a heterozygote? The absence of phenotypically *Lac*+, balanced heterozygotes, in the previous crosses gave a correct hint. A V_6^r *Het B–M–m53* stock was available, and crossed with the *T–L–*B_1–Lac_{1a}–^{st112}. Several V_6 *r/s* diploid prototrophs were recovered (via the dominance of V_6^s) and characterized as segregating for *Lac*–^{m} V_6^r/–st V_6^s. These heterozygotes were phenotypically lactose-negative, although they gave rise to a profusion of *Lac*+ papillae on further incubation on EMB lactose, probably as a result of segregation and crossing over. On the other hand, the same cross gave the usual low percentage of *Lac*+ colonies, and now that *Het* was present a few were *Lacv*. However, these were segregating +/–, and *not* balanced. They are presumed to be *Lac*+ crossovers, which have become involved in nondisjunctions in the usual proportions under the influence of *Het*. Stringent selection for such crossovers was exercised, and their secondary incorporation in a heterozygote was an incidental feature of *Het* action. The +/– types are probably *Lac*++/*m87*– *st112*–; this is not certain, but at any rate all of them segregated only stable *Lac*– and *Lac*+.

We have here a position effect which is closely parallel to the lozenge case analysed by Green and Green (1949). The +–/–+ compound is ineffective in contrast to the full effect of the haploid ++ and the diploids +–/++; –+/++ and ++/–– (?). Some of the down shifts in mutability in *m87*– may be due to mutation at Lac_{1a}; one st– derived from *m87* lost the potentiality to recombine with *st112*. A third locus affecting lactose, Lac_4, is not far away either, but Lac_4– shows complementary action at least with *m53* to produce balanced *Lac*+ heterozygotes. About 200 other mutants belonging to this complex await further study.

A second allelic series at the Lac_3 locus has received more physiological than genetic study. The pleiotropic effect (*Lac– Mal– Glu–*) can be tied to a single locus beyond reasonable doubt: the effect has recurred several times in the same form; all of the *Glu*– so far isolated proved to be Lac_3–; reverse mutations are readily selected on any one sugar, and are homotypic for all three if they involve the Lac_3 locus; an "intermediate," temperature-sensitive allele has occurred twice (Lac_3t) which shows a graded effect on all three characters. On the other hand, Lac_3t shows best of all that the pleiotropism is not a trivial interaction in terminal metabolism, for at different temperatures, the phenotypes *Lac– Mal– Glu–*;

−++, and +++ are found. In addition, the specific enzymatic adaptation of *Lac*+ to the disaccharides provides for cell suspensions with the phenotypes −−+, −++, and +−+. Whatever the ultimate basis of the pleiotropic effect may prove to be, it is concerned with the formation rather than the action of these enzymes, and as such stands in flat contradiction to that aspect of the one-gene—one-enzyme generalization which was not at issue in connection with the multigenic control of a single enzyme. However, I think there can be no question but that the most fruitful working hypothesis in any phenogenetic analysis is that a single primary effect is involved, as is already suggested by the range of the Lac_3t mutant. This primary effect cannot be regarded as at the overt enzymatic level here, but rather in or beyond the experimentally almost inaccessible realm of the *cellular* (i.e., not genic) mechanisms of enzyme synthesis.

The point of view expressed above is specifically supported by experiments on the interplay of genetic and environmental factors on the formation of lactase. The effect of the Lac_1− (and Lac_{1a}−) mutations in altering the adaptive responsiveness of the cell to lactose, rather than the substrate specificity of the lactase, was mentioned before. As a second example, the analogue neolactose (altrose-galactoside) also reacts with lactase but does not stimulate its "adaptive" formation by *Lac*+. Attempts to select for mutations which would sensitise the cell to neolactose led instead to one which produced lactase constitutively—that is, to at least as large an extent, and perhaps larger, in cells grown on glucose as compared to lactose. A third datum is that Lac_3t phenotypes were based on temperature thresholds for formation of the enzymes, rather than their action. Every mutational effect we have been able to analyse, therefore, has had no effect on the action, but only on the formation of lactase. The autonomous action of alleles would be the best criterion for directness of gene action, but so far no examples can be cited in microbial genetics.

When Lac_3− is grown on EMB agar containing lactose, maltose, or glucose a number of homotypic mutations appear and are selected for. Most of these are true reverse mutations (by subsequent crossing tests) with the phenotype +++. A smaller proportion, however, show phenotypes with every permutation of +/− for these three sugars, except that −−+ so far isolated have all been weak fermenters of glucose. In addition to the "specific suppressors," +−− and −+−, the type ++− can also be selected either on lactose or maltose. One of the +−− types proved to be the mutation for constitutive lactase referred to earlier.

The phenotype −+− (lactose−; maltose+; glucose−) is especially interesting to the biochemist for it points to a mechanism of maltose metabolism more complex than the usually assumed simple hydrolysis to two moles of glucose. Fortunately, M. Doudoroff and his colleagues at the University of California at Berkeley undertook to lend their skill to the biochemical analysis of this curiosity. Eventually, it was discovered that maltose was metabolized by a dismutative polymerization to starch: n(maltose)⟶$(glucose)_n$ + n glucose, following which the starch was phosphorolysed, for the most part bypassing free glucose: $(glucose)_n$ + (n-1) H_3PO_4 ⟶ (n-1) glucose-1-phosphate + (1) glucose, (Doudoroff *et al.*, 1949). Meanwhile, Monod and Torriani (1950) had come to the same conclusion, without the benefit of a glucose-negative mutant, and named the polymerase "amylomaltase." The present result is that the mutant has posed a weightier problem than it perhaps helped to solve, for we are left with a neat paradox in the form of the n glucose molecules in the first equation above. The biochemist's scheme works very well for dried cell preparations, and the n(glucose) accumulates as anticipated. Intact cells, however, utilize maltose completely, with no trace of residual glucose. If glucose is added to a maltose-metabolizing system, however, added glucose is untouched. A number of untested hypotheses have come to mind, especially that a phosphorylation precedes the dismutative polymerization of maltose, but the more immediate question of the basis for the failure to utilize free glucose has not been settled. This sort of impasse is not unique in biochemistry, but it has come to be especially characteristic of the application of genetic and adaptive enzyme analyses.

In other studies, a series of alleles has been established as the basis of streptomycin resistance in *E. coli* K-12 by Newcombe and Nyholm (1950b) and Demerec (1950). The major allelic states are characterized as type sensitive, S^s; resistant, S^r; and S^d, dependent. A number of quantitative variations of resistance or dependence have been mentioned, but at the time of this writing, no data have been published to determine whether these are due to modifiers or further allelic changes. If S^d cells are plated on non-streptomycin medium, mutations to types resembling S^s and S^r can be selected. These may include both interallelic shifts and modifier mutations at other loci.

One locus accounts for most or all of the mutations leading to streptomycin-resistance. By contrast, resistance to chloromycetin is polygenic, as shown by Cavalli and Maccacaro (1950). Crosses between a stock whose resistance to chloromycetin had been increased to a high level by repeated selection and the type sensitive led to a segregation of a wide range of levels of resistance. Furthermore, there was a correlation between the level of resistance of a prototroph and the proportion of unselected markers derived from the resistant parent. The recombinational investigation of these two cases of oligo- and polygenic effects respectively thus accords closely with the concepts of single- and multi-step resistance formulated by Demerec (1948).

Two examples of genic instability should be mentioned before we leave the discussion of allelic relationships. Mutation rates as high as 10^{-2} per division can be detected and distinguished from phenotypic variegation in bacteria, but relatively few examples have been studied (Zelle, 1942; Bunting, 1946). One of our mutable stocks, W-716, arose as a phenotypic reversal to *Lac+* from a $Lac_1{}^{-st}$ plated on EMB lactose. This *Lac+* repeatedly throws a spectrum of types, from Lac^{-st} and Lac^{-m} of all grades through slow lactose-fermenters to types barely distinguishable from W-716. The Lac^{-m} types reverted at relatively low frequencies to *Lac+* showing the same range of instability. The instability makes it difficult to distinguish between mutants and recombinants in critical crosses. However, the occurrence of Lac^{-st} from the derived $Lac^{-m} \times$ type makes it likely that the instability involves a suppressor locus.

The second example was detected as a variegated colony from a UV plating of *Mal+* on EMB maltose. Sectored colonies are very common at the initial occurrence of fermentation mutants, probably due to the presence of several nuclei per bacterial cell, together with the disturbances induced by UV, but the sectoring ordinarily disappears after a single streaking-out. In this case, variegated colonies were noted throughout serial streakings. The *Malv* behaves like the unstable *Lac+* just reviewed, except that a few apparently stable *Mal+* have been isolated. As a rule, the cycle is continued: Mal+ ⇄ Mal slow ⇅ ⇄ Mal− the rates corresponding to each arrow varying considerably from culture to culture.

The curious conclusion derived from these cases is that instability at a locus may persist throughout allelic substitutions. Most of the *Mal+* isolated as reversions from even the more stable *Mal−* forms in the series proved to be highly unstable. In many examples of genic instability, this attribute is assignable to a specific allele (e.g., *dottable* in maize), and is lost when the allele shifts to another state, but this type of instability has not been found in our material. It should be pointed out that almost all of the UV-induced mutations in our studies have involved sharp transitions from one state to another, with no evidence of transient or persistent instability. Many of the mutants allow of reverse-mutation and can scarcely be regarded as deletions (most of which should be inviable anyhow in a haploid organism). Comparative studies with X-rays and nitrogen mustard would be desirable.

"Extranuclear Heredity"

To this point, the genetic changes discussed have been presumably nuclear, and no mention has been made of the possibility of extranuclear agents in bacterial heredity. Experimental progress in cytoplasmic genetics will have been summarized by other speakers here. The three ways in which cytoplasmic effects would be most likely to be recognized in material like *E. coli* K-12 are: a) the physical separation and experimental extranuclear transfer of the agent; b) kinetic evidence for induced loss or attenuation; or c) a failure of segregation and of ratio-reversal in reversed crosses. Barring the discovery of specific agents comparable to acriflavine on yeasts cytoplasmic effects, if any, are most likely to be found in crosses between independently isolated strains rather than as the discrete mutations of the previous discussion.

Nevertheless, one character of *E. coli* K-12 can be regarded as subject to extra-nuclear hereditary control, namely, lysogenicity. The principal features and the genetic importance of this phenomenon, which consists of a symbiotic, intracellular association of bacterium with a virus were pointed out many years ago (Burnet and Lush, 1936).

Our first experience of lysogenicity was with *Salmonella typhimurium*, where it appears to be nearly ubiquitous (Boyd, 1950). Our interest was accentuated by the accidental discovery that *E. coli* K-12 was lysogenic. This resulted from the occurrence of a sensitive "mutant" as a survivor of UV treatment. The stock was mixed with type

cells for other purposes, and we were surprised to find numerous phage plaques. Contamination with extraneous phage was first suspected, but we soon showed that the phage was carried by all of our stocks except for the unique sensitive strain. The occurrence of the latter was entirely fortuitous, but some of the distinct *E. coli* strains studied for the purposes described in the next section are also sensitive to this phage, and would have served as independent indicators for its discovery. *E. coli* K-12 has been studied as a bacteriological type for nearly 30 years with no hint of its lysogenic character—an eloquent commentary on the latency of its symbionts.

The exposure of sensitive cells to suspensions of the free phage, which we named "λ," by analogy to a killer factor in *Paramecium*, results in the lysis of a variable proportion of cells. The survivors include sensitives, new lysogenics, and an immune type which is genetically resistant to λ, but does not carry it. The genetic relationships of these types are under study now. Sensitives can be crossed with each other, and the transfer of lambda from a lysogenic to a previously sensitive culture is not associated with alterations of any other markers. The speculation that λ might be involved in genetic recombination needs no further mention. In addition to mutations of the host bacterium, we have noted mutations of the symbiont to a form which attacks the standard lysogenics, but does not, however, evoke lysogenicity itself.

So far, no marked changes in the phenotype have been found in association with lysogenicity. It is anticipated, however, that the latent virus alters the serological character of the cells at least to the extent of the phage-antigens themselves. In addition, Lwoff has found that, under certain conditions, the latent phage of *Bacillus megatherium* can be activated by UV so as to provoke lysis (Lwoff, *et al.*, 1950). Lwoff and Delbrück (private communication) have extended this observation to K-12, so that infection with λ can be regarded as a transformation to high UV-sensitivity. The natural history and phylogeny of the transforming agents of pneumococcus, *Hemophilus*, and possibly other bacteria are not likely to be uncovered in the near future, but one possibility that deserves close scrutiny is that they are akin to latent viruses whose lytic activity is no longer discernible. As Altenburg (1946) and others have pointed out, the genes of both partners in a symbiosis are available for mutations affecting the adaptation of the complex. The converse, that an adapted symbiont, i.e., a plasmagene, might become virulent has also been postulated. In the absence of any direct evidence against either view, there is no harm in suggesting that both processes take place. Instead of debating moot questions on the taxonomy of viruses and plasmagenes, we should encourage the current trend of emphasis on the extraction of genetically useful information from virology and chemotherapy as well as the more orthodox disciplines of cell behavior.

Ability of New *E. coli* Strains to Cross

The recombination studies thus far summarized have all involved mutants derived from a single strain, K-12 of *E. coli*, and for the most part derived from the two polyauxotrophs 58-161 $B-M-$ and $Y-10\ T-L-B_1-$. Every other auxotroph mutant from K-12 (excepting certain pantothenicless– W. Maas, personal communication) has reacted in the same way, but this pair was chosen as giving the highest yields of prototrophs, probably owing to linkage relationships.

Attempts to find recombination in other strains were at first uniformly unsuccessful, including tests on strains W (B. D. Davis, personal communication), B, L-15, and several others. Cavalli and Heslot (1949) then reported that culture "123" of the British National Type Culture Collection could be crossed with WG-1 (a generic term for all cultures derived from strain K-12). "123" was received as a complex auxotroph whose nutrition could not be satisfactorily analysed either by Cavalli or ourselves, and for this reason, mainly, is not very suitable for more extensive work. In general, the procedure for determining crossability by the use of auxotroph mutants is too laborious to be worth applying when the probability of success is as low as it proved empirically to be. Fortunately, a simpler screening method has been devised (Lederberg, 1951) that has provided satisfactory solution to one aspect of this problem. The main difficulty had been that each new culture had and still has to be subjected to painstaking procedures of isolating two, non-overlapping, diauxotrophic mutants before intra-fertility of the strain could be tested. By a combination of streptomycin resistance and prototroph selection (SRP), however, new strains can be tested for inter-fertility with WG-1 with a minimum of individual manipulation. For this purpose, the new strains must be streptomycin-sensitive and should be prototrophic, $S^s\ X+$ (as most *E. coli* proves to be). WG-1 tester (usually W-1177) is

streptomycin-resistant and polyauxotrophic S^r X^-. Thus, neither the tested nor the tester strains will form colonies on a minimal streptomycin medium which selects only the combination S^r $X+$. This genotype will occur as the result of recombination of the two strains or, occasionally, by mutation of S^s to S^r. Fortunately, this mutation is one of the least frequent known. The occurrence of recombination can be verified by comparing the unselected markers of the SRP with the parents, but the main purpose of the procedure is to screen out the most likely candidates for further study. In practice, the two strains are inoculated in broth and allowed to grow together overnight. They are then harvested, and about 5×10^8 cells plated on EMS maltose agar to which 100 micrograms/ml of streptomycin have been added. The strains which consistently produce SRP, especially if they are segregating *Mal*+ and −, are retained for closer study.

About 650 cultures have been tested in this way, each from a separate individual, mostly human. (We are indebted to the staff of the Wisconsin Public Health Laboratory, to Dr. C. P. Miller and Miss M. Bohnhoff, and especially to R. S. Benham and his staff at the University of Chicago Hospitals for supplying the larger part of these cultures.) About 25 of them have shown signs of recombination with WG-1; at least 20 of them almost certainly. Polyauxotroph mutants have been prepared in WG-1 through -4, and have been used in the demonstration that recombination is successful in all inter- and intra-strain combinations of these strains.

Culturally, all of the WG-1 fertile strains conform to the description of *E. coli*, or possibly of intermediates, although a considerable number of aerogenes-type, cellobiose-fermenting cultures have been included in the tests. A variety of somatic antigen serotypes are included (we are indebted to Dr. W. H. Ewing of the USPHS Communicable Disease Center for carrying out some of these serological determinations). Their genetic diversification has been reflected also in the fermentation of lactose and of sucrose, and particularly in patterns of sensitivity to phages, including λ, and to antibiotics produced by various other *coli* strains, (colicins). WG-2 produces a colicin active on most of the others. It appears likely that many potentially compatible combinations may fail owing to the suppression of WG-1 by a colicin produced by the other parent. We are preparing for a detailed comparative genetic and serological study of these and additional strains. So far, the main point is that cross-fertile strains do exist in respectable numbers, so that K-12 is not a unique representative of bacterial recombination. Nevertheless, Professor Tatum and I are willing to admit our very good luck in his choice of a fertile strain for the first experiments.

One aspect of the problem that is unfortunately not encompassed by the SRP selection method is the existence of other compatibility groups in *E. coli*. For this objective, there is no alternative but to continue the isolation of suitable mutants from each culture. However, a distinct recombination system has been found in another distantly related species, *Salmonella typhimurium*. It should be emphatically stressed at the outset of this discussion that this system has already shown a number of unique features *not* shared by *E. coli* WG-1, and that owing to the preliminary character of the work our conclusions may have to be modified as new information is accumulated.

Genetic Recombination in *Salmonella*

Evidence for recombination in *Salmonella* serotypes has been sought in terms of the occurrence of prototrophs in combinations of auxotroph cultures. After inconclusive results in a few trials with various species (*S. coli*, *S. poona*, *S. madelia*, *S. cholerae-suis*) it was decided to make a concerted survey of a coherent set of *Salmonella typhimurium*. In order to avoid unnecessary duplication, the 22 phage-resistance types described by Lilleengen (1948) and kindly provided by him, were used as representative of the species. Diauxotroph mutants have been obtained with the help of the penicillin selection method in 20 of these 22 strains; two were refractory. Of the 200 possible intra- and inter-strain combinations, 99 have been tested, and nine more or less consistently produced prototrophs on minimal agar, while control platings of the separate parents were barren. One combination gave exceptionally high yields, and our attention has been focused on this pair: LT-2A, a methionine-histidineless mutant (*M-H-*) from Lilleengen's type 2, and LT-22A, a two-step mutant from type 22 requiring phenylalanine plus tryosine, and tryptophane. Neither 2A nor 22A has produced any prototrophs even in dense individual platings, but together will produce as many as 10 prototrophs per million parental cells inoculated. In most of the experiments a Gal− Xyl− derivative of 22A has been used to provide two unselected markers. The great majority of the prototrophs from 2A × 22A were Gal− Xyl−; however, a very small number of the other combinations have also been found.

In one comparison of the *Salmonella* and *E. coli* systems, Davis' (1950b) filtration experiment was duplicated. A U-tube, with a sintered glass sterile filter plate in the cross-limb, was filled with broth and the two compartments inoculated with 2A and 22A respectively. From time to time, the liquid was flushed back and forth between the compartments by alternating suction. When the broth was saturated, the cells from each compartment were harvested, washed and plated separately on minimal agar. It was repeatedly found that about 10^{-7} prototrophs appeared from the 22A, but none from the 2A culture. Control experiments in which only one compartment was inoculated verified the integrity of the filter.

This experiment showed that, in contrast to *E. coli* combinations, a filtrable agent (FA) was produced by 2A that reacted with 22A to produce prototrophs. However, filtrates prepared directly from 2A were inactive. The paradox was resolved when it was found that the addition of a 22A filtrate, or of a lysate of 2A originated from a lysogenic phage secreted by 22A, provoked the formation of FA by 2A. FA, then, is not a normal component of 2A, but is produced under the stimulus of a latent phage. We have not succeeded in extracting significant FA activity from 2A cells heat-killed, dried, or autolysed under conditions which do not destroy FA activity.

FA is resistant to a variety of disinfectant treatments that sterilize the bacteria: exposure to 56°C for 30 minutes, precipitation with alcohol, or shaking with chloroform or benzene. It can be concentrated by precipitation with ethanol or ammonium sulfate, or by sedimentation in a high speed centrifuge. A linear assay of FA is available from the yield of prototrophs produced by mixing the sample with $10^9 - 10^{10}$ cells of 22A on minimal agar plates, throughout the range of 10 to 1000 prototrophs per plate. The most potent preparations have an activity of about 10^4 units/ml. (One unit is equivalent to the production of one prototrophic cell.) In a preliminary test, the activity was not altered by DNAse.

Our suspicion that FA might be related to the filtrable granules of L-type cultures (reviewed by Klieneberger-Nobel, 1951) led to a test of various agents known to provoke the L-form for their ability to evoke FA. Aging in broth, lithium chloride, crystal violet, and especially penicillin were successfully used in place of the stimulus from 22A to evoke FA from 2A. The concentration of penicillin used, 1 U/ml. does not appreciably inhibit growth, but some abnormal cell forms, including swollen filaments, are seen. The morphological details of the origin of FA remain to be worked out.

FA can be manifested in several ways. Prototrophs are induced from at least five other diauxotrophic cultures, each from a different *Salmonella* strain, and encompassing requirements for phenylalanine, cystine, leucine, threonine, isoleucine + valine, pyrimidines, and purines. However, platings with mutants of other strains, or with *E. coli* W-1177, have not given prototrophs. In addition platings of T22A *Gal– Xyl–* with FA on EMB with galactose plus xylose results in two types of papillate growth which give rise to *Gal– Xyl+* and *Gal+ Xyl–*, respectively. So far, each genetic factor has tended to change independently. At any rate, with very few exceptions, only those character alternatives for which selection was exerted have been recovered, and very little concomitant recombination of unselected markers (as in *E. coli*) has been seen so far. Three possible interpretations may be discussed: 1) FA may induce a non-specific genetic instability in susceptible bacteria, leading to the formation of a variety of types including those selected for. 2) FA may consist of a population of specific, distinct, transforming factors, corresponding to the genotype of the donor cells, each acting independently of the other. 3) FA may be a uniform factor, acting differently in different cells, for example, as a gamete would depending on patterns of recombination and crossing-over after fertilization. The distinction between 1) and 2) will depend largely upon the isolation and use of LT-2 mutants with distinctive markers; between 2) and 3) on further studies of recombination of selected and unselected markers. The possibility must be kept in mind that the treatments required to sterilize FA may result in or select for artefacts not typical of the normal recombination pattern.

As a working hypothesis, we suggest that FA can be correlated with the granular phase of L-type colonies. Klieneberger-Nobel reported some years ago that a "pleuropneumonia-like organism" was associated with cultures of *Streptobacillus moniliformis*, but regarded this "L-type" as a symbiont or parasite. However, she has more recently accepted Dienes' vigorously documented proposition that the L form is a stage in the life history of this bacterium. Dienes, meanwhile, has shown that L-forms, in various aspects, can be elicited from a variety of bacterial species, especially with the aid of penicillin. These forms can be propagated on horse-serum agar; according to Tulasne *et al.*, (1950), lactoflavine will re-

place the requirement for serum. In a few cases, especially with Proteus, the cultures will revert to the normal bacterial form. The morphology, and especially the terminology, of the L– cultures are confusing, but in general the picture seems to be that tiny granules, about 0.2 – 0.3 microns in diameter are produced, either directly from bacteria, or via swollen large bodies. The latter are characteristic of L-cultures, but their usual fate is lysis. In some cases, however, they have been reported to become converted into cysts of granules or of bacteria. There has been no agreement as to the functions of the L-forms, although there have been persistent suggestions that they may have something to do with sexuality. Several authors describe large bodies as forming from the conjunction of two or more cells, but relevant genetic studies are lacking.

The evidence connecting our FA with the L-forms of other workers as hastily summarized above is suggestive rather than conclusive. FA preparations contain tiny "granules," scarcely resolvable with the phase contrast microscope. These granules follow FA in sedimentation. Rabbit O-antiserum against *S. typhimurium* agglutinates the granules together with FA. Incubation of FA with antiserum broth results in the formation of large bodies from the clumps of granules, in an interval of about 4 hours, Figure 11; (bacterial cells were added to the system to provide a standard of size and form—they have not been recovered from otherwise sterile FA preparations.) The production of large bodies, and the effectiveness of penicillin and of phage in provoking the granules tend to relate this system to those described by Dienes and others. However, we have not yet succeeded in propagating the granules, or in obtaining growth of FA activity, but we have not yet adequately imitated the design of Dienes' experiments in soft agar.

Our FA filtrates have been tested for sterility by prolonged incubation of small and large samples inoculated into Penassay broth, with and without bovine serum, observed for several weeks with no trace of bacterial turbidity. FA-containing filtrates are difficult to sterilize, and we have established the practice of passing the clear supernatants first through a medium, then a fine Mandler filter, and then heating the filtrates to 58 degrees for 30 minutes. Any single one of these treatments is adequate to sterilize an ordinary broth culture. There is an unmistakable suggestion of "filtrable" elements which can regenerate bacteria probably along the lines of the reversion of L-type cultures as described by several other workers. The conditions of dormancy and of reversion to bacteria are too poorly understood for more detailed discussion.

It must be quite obvious that the genetic effects of FA appear, at this time, to duplicate the well-known pneumococcus transformation. While casual published statements concerning the size of the transforming principle may rule out the participation of L-granules in that system, the possibility that they play a part in other transformations should be examined very carefully. It is very likely that criteria of sterility which do not take into account the filtrability, dormancy, and resistance of L-granules may lead to artefacts, especially if growing cells of a receptive strain help to provide the obscure conditions which favor the reversion of L-forms to bacteria. The implications of the L-system for any problem in which sterility is a decisive issue—and there are few bacteriological problems where it is not—are plainly to be seen.

The artefact just mentioned, reversion from L to bacteria, has no special genetic interest, but adds one more reason for studying transformations with more than one marker at a time. In *Salmonella*, we have not succeeded in reisolating the donor 2A from interactions of FA with 22A under conditions which would allow it to be distinguished from the selected type. For example, platings on methionine-histidine agar gave only prototrophs, and no *M–H–* like the bacterial source, and the same holds for the *Gal* and *Xyl* differential markers.

There may be a contradiction between associating FA with a gametic phase that can be propagated in some systems or circumstances as a living organism, and the peculiar character of the range of recombination types so far recognized. This will have to be settled by further experiments. The unproven possibility that the filtered and sterilized FA may be degraded has already been mentioned. In addition, recombination may be hindered by structural differentiation between the various strains used in these experiments.

In order to establish clearly the unique features of the *E. coli* system of recombination, we have previously emphasized the very pronounced differences between it and various interpretations of pneumococcus transformation. *Salmonella* shows some features in common to both. Muller (1947) made a very reasonable synthesis in his interpretation of the pneumococcus transformation: "there were, in effect, still viable bacterial chromosomes, or parts of chromosomes floating free in the medium used. These might, in my opinion, have penetrated the capsuleless bacteria and in

part at least taken root there, perhaps after having undergone a kind of crossing over with the chromosomes of the host." To the time of this writing, the genetic exchanges in the pneumococcus system have involved single characters at each step. No attempt has been made to ascertain whether more complex exchanges might not occur between intact cells, so that it has been difficult to correlate the different studies. By now, the time has arrived to determine whether the apparently conflicting data from different methods and sources can be assimilated into a unified concept of bacterial heredity.

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REFERENCES

ALEXANDER, H. E., and LEIDY, G., 1951, Determination of inherited traits of *H. influenzae* by desoxyribonucleic acid fractions isolated from type-specific cells. J. Exp. Med. *93*: 345–359.

ALTENBURG, E., 1946, The symbiont theory in explanation of the apparent cytoplasmic inheritance in *Paramecium*. Amer. Nat. *80:* 661–662.

ATWOOD, K. C., 1950a, The role of lethal mutation in the killing of *Neurospora* conidia by ultra-violet light. Genetics *35*: 95–96.

1950b, The homology patterns of induced lethal mutations in *Neurospora crassa*. Biol. Bull. *99:* 332.

ATWOOD, K. C., and NORMAN, A., 1949, On the interpretation of multi-hit survival curves. Proc. Nat. Acad. Sci. Wash. *12*: 696–709.

BISSET, K. A., 1950, The Cytology and Life-history of Bacteria. Edinburgh, E. and S. Livingstone.

BOYD, J. S. K., 1950, The symbiotic bacteriophages of *Salmonella typhi-murium*. J. Path. Bact. *52*: 501–517.

BRAUN, A. C., and ELROD, R. P., 1946, Stages in the life history of *Phytomonas tumefaciens*. J. Bact. *52*: 695–702.

BUNTING, M. I., 1946, The inheritance of color in bacteria, with special reference to *Serratia marcescens*. Cold Spring Harb. Symposium Quant. Biol. *11*: 25–32.

BURNET, F. M., and LUSH, DORA, 1936, Induced lysogenicity and mutation of bacteriophage within lysogenic bacteria. Austr. J. Exp. Biol. Med. Sci. *14*: 27–38.

BURNHAM, C. R., 1948, Cytogenetic studies of a translocation between chromosomes 1 and 7 in maize. Genetics *33*: 5–21.

BUZZATI-TRAVERSO, A., VISCONTI, N. di M., and CAVALLI, L. L., 1948, Polyploidy in bacteria? Nature, Lond. *162*: 295.

CAVALLI, L. L., 1950, La sessualita nei batteri. Boll. Ist. sierotera Milano *29*: 1–9.

CAVALLI, L. L., and HESLOT, H., 1949, Recombination in bacteria: outcrossing *Escherichia coli* K-12. Nature, Lond. *164*: 1057.

CAVALLI, L. L., and MACCACARO, G. A., 1950, Chloromycetin resistance in *E. coli*, a case of quantitative inheritance in bacteria. Nature, Lond. *166*: 991–992.

CLARK, J. B., HAAS, F., STONE, W. S., and WYSS, O., 1950, The stimulation of gene recombination in *Escherichia coli*. J. Bact. *59*: 375–379.

COHN, M., and MONOD, J., 1951, Purification et propriétés de la beta-galactosidase (lactase) d' *Escherichia coli*. Biochim. Biophys. Acta *7*: 153–174.

DAVIS, B. D., 1950a, Studies on nutritionally deficient bacterial mutants isolated by means of penicillin. Experientia *6*: 41–50.

1950b, Nonfiltrability of the agents of genetic recombination in *Escherichia coli*. J. Bact. *60*: 507–508.

DEMEREC, M., 1948, Origin of bacterial resistance to antibiotics. J. Bact. *56*: 63–74.

1950, Reaction of populations of unicellular organisms to extreme changes in environment. Amer. Nat. *84*: 5–16.

DEMEREC, M., and FANO, U., 1945, Bacteriophage-resistant mutants in *Escherichia coli*. Genetics *30*: 119–136.

DEMEREC, M., and LATARJET, R., 1946, Mutations in bacteria induced by radiation. Cold Spring Harb. Symposium Quant. Biol. *11*: 51–59.

DOUDOROFF, M., HASSID, W. Z., PUTMAN, E. W., POTTER, A. L., and LEDERBERG, J., 1949, Direct utilization of maltose by *Escherichia coli*. *J. Biol.* Chem. *179*: 921–934.

FISHER, R. A., 1947, The theory of linkage in polysomic inheritance. Philos. Trans. B. *233*: 55–87.

GREEN, M. M., and GREEN, K. C., 1949, Crossing-over between alleles at the lozenge locus in *Drosophila melanogaster*. Proc. Nat. Acad. Sci. Wash. *35*: 586–591.

HAMLETT, G. W. D., 1926, The linkage disturbance involved in the chromosome translocation I. of *Drosophila*, and its probable significance. Biol. Bull. Vol. *51*: 435–442.

KLIENEBERGER-NOBEL, E., 1951, Filterable forms of bacteria. Bact. Rev. *15*: 77–103.

LAUGHNAN, J. R., 1949, The action of allelic forms of the gene A in maize. Proc. Nat. Acad. Sci. Wash. *35*: 586–591.

LEDERBERG, E., 1948, The mutability of several Lac-mutants of *Escherichia coli*. Genetics *33*: 617.

1950, Genetic control of mutability in the bacterium *Escherichia coli*. PhD. thesis. Univ. of Wisconsin.

LEDERBERG, J., 1947, Gene recombination and linked segregations in *Escherichia coli*. Genetics *32*: 505–525.

1949, Aberrant heterozygotes in *Escherichia coli*. Proc. Nat. Acad. Sci. Wash. *35*: 178–184.

1950a, The selection of genetic recombinations with bacterial growth inhibitors. J. Bact. *59*: 211–215.

1950b, Isolation and characterization of biochemical mutants of bacteria. Meth. Med. Res. *3*: 5–36.

1950c, The Beta-D-galactosidase of *Escherichia coli*, strain K-12. J. Bact. *60*: 381–392.

1951, Prevalence of *Escherichia coli* strains exhibiting genetic recombination. Science *114*: 68–69.

LEDERBERG, J., and TATUM, E. L., 1946, Novel genotypes in mixed cultures of biochemical mutants of bacteria. Cold Spring Harb. Symposium Quant. Biol. *11*: 113–114.

LILLEENGEN, K., 1948, Typing of *Salmonella typhi murium* by means of bacteriophage. Acta. Path. Microb. Scand. Suppl. *77*.

LONGLEY, A. G., 1945, Abnormal segregation during megasporogenesis in maize. Genetics *30*: 100–113.

LURIA, S. E., 1946, Spontaneous bacterial mutations to resistance to antibacterial agents. Cold Spring Harb. Symposium Quant. Biol. *11*: 130–138.

LWOFF, A., SIMINOVITCH, L., and KJELGAARD, N., 1950, Induction de production de phage dans une bactérie lysogène. Ann. Inst. Pasteur. *79*: 815–858.

MCCARTY, M., TAYLOR, H. E., and AVERY, O. T., 1946, Biochemical studies of environmental factors essential in transformation of pneumococcal types. Cold Spring Harb. Symposium Quant. Biol. *11*: 177–183.

MITCHELL, M. B., and MITCHELL, H. K., 1950, The selective advantage of an adenineless double mutant over one of the single mutants involved. Proc. Nat. Acad. Sci. Wash. *36*: 115–119.

MONOD, J., and TORRIANI, A. M., 1950, De l'Amylomaltase d' *Escherichia coli*. Ann. Inst. Past. *78*: 65–77.

MULLER, H. J., 1947, The gene. Proc. Roy. Soc. Lon. B. *134*: 1–37.

NELSON, T. C., 1951, Kinetics of genetic recombination in *Escherichia coli*. Genetics *36*: 162–175.

NEWCOMBE, H. B., and NYHOLM, M. H., 1950a, Anomalous segregation in crosses of *Escherichia coli*. Amer. Nat. *84*: 457–465.

1950b, The inheritance of streptomycin resistance and dependence in crosses of *Escherichia coli*. Genetics *35*: 603–611.

RHOADES, M., 1942, Preferential segregation in maize. Genetics *27*: 395–407.

STEMPEN, H., 1950, Demonstration of the chromatinic bodies of *Escherichia coli* and *Proteus vulgaris* with the aid of the phase contrast microscope. J. Bact. *60*: 81–87.

STEMPEN, H., and HUTCHINSON, W. G., 1951, The formation and development of large bodies in *Proteus vulgaris* OX-19. J. Bact. *61*: 321–335; 337–344.

STERN, C., 1936, Somatic crossing-over and segregation in *Drosophila melanogaster*. Genetics *21*: 625–730.

STEPHENS, S. G., 1948, A biochemical basis for the pseudo-allelic anthocyanin series in *Gossypium*. Genetics *33*: 191–214.

TATUM, E. L., 1945, X-ray induced mutant strains of *Escherichia coli*. Proc. Nat. Acad. Sci. Wash. *31*: 215–219.

TATUM, E. L., and LEDERBERG, J., 1947, Gene recombination in the bacterium *Escherichia coli*. J. Bact. *53*: 673–684.

TAYLOR, H. E., 1949, Additive effects of certain transforming agents from some variants of pneumococcus. J. Exp. Med. *89*: 399–424.

TULASNE, R., MINCK, R., and MULLER, L., 1950, Technique pour la culture des formes submicroscopiques (formes L) du *Proteus vulgaris* en milieu liquide. C. R. Acad. Sci. *230*: 152–154.

WITKIN, E. M., 1947, Genetics of resistance to radiation in *Escherichia coli*. Genetics *32*: 221–248.

ZELLE, M. R., 1942, Genetic constitutions of host and pathogen in mouse typhoid. J. Infect. Dis. *71*: 131–152.

ZELLE, M. R., and LEDERBERG, J., 1951, Single cell isolations of diploid heterozygous *Escherichia coli*. J. Bact. *61*: 351–355.

DISCUSSION

ATCHLEY: During the past year numerous experiments have been performed in our laboratory in an attempt to demonstrate transformation in the colon bacillus.

The strains which we have tried to transform were derived from *E. coli* B, B/R, W, and K12. The markers we have tried to transfer to appropriately deficient organisms have included (1) the ability to synthesize certain nutrients, and (2) resistance to bacteriophage T_1. The cultural environments used for these experiments have included a synthetic broth, Medium A, and two different types of a neopeptone-meat infusion broth. Beef serum albumin has been added in some of the experiments as a source of serum factor.

From cultures of organisms showing the character we wished to transfer to receptor organisms we have sought active transforming agents by making the following types of preparations: (1) a relatively pure, lightly polymerized DNA, (2) crude lysates made from cells broken up by repeated freezing and thawing, (3) crude lysates made by treating cells with ultrasonic vibration, and (4) filtrates of cultures in which the presumed "donor" strain has grown. In no case were we able to show that the preparations we used could increase the rate at which treated strains mutated from auxotrophism to prototrophism or from T_1 sensitivity to T_1 resistance.

DELAMATER: I would merely like to note the essential similarity of many of the nuclear figures presented by Dr. Lederberg to those which were described in a previous paper.

MULLER: The discussion of Dr. Lederberg's paper reminds me of the time, early in the history of the *Drosophila* work—about 1913 to 1921—when we were criticized for trying to present so appar-

ently mechanical a scheme. It was asserted by Bateson and his group, as well as by others, that we were able to make it fit only by bolstering it up with various accessory *ad hoc* hypotheses of a special nature, such as differential viability, multiple factors, interference, non-disjunction, etc., although these phenomena were in fact of just the sort to be expected if the scheme were true. Fortunately this criticism did not discourage the *Drosophila* workers from pushing their analyses still further along the same lines, which they saw were yielding results, and so they were enabled to obtain ever more convincing evidence of the validity of the so-called special hypotheses, as well as of the general scheme, and at the same time to uncover deeper-lying problems. In the same way, it seems to me, the present genetic analyses of *E. coli* by Dr. Lederberg deserve to be pushed still further, until the genetic scheme becomes quite clear, rather than abandoned now in favor of excursions into more mysterious waters. For this methodical procedure will provide both the conceptual and the biological tools whereby the attack on the more recondite problems of gene physiology can finally be pressed home with far greater effectiveness.

In regard to one of the puzzling features of the genetic mechanism disclosed by Lederberg's studies, that of a single-branched linkage map resembling one based on a heterozygous translocation, it would at first sight appear as if this implied the presence of different mating types. For if this really represents a translocation, as the beautiful linkage results indicate, we should have to suppose that the two cells which unite always differ in regard to this translocation. However, in view of Lederberg's evidence that individuals of the same clone can cross, a different interpretation is needed.* A simple one would be provided by supposing that the haploid contains two non-homologous chromosomes, and that after fusion of haploid cells, at meiosis, the chromosomes derived from the same parent are in each case held together at a given point or region. This may well be a heterochromatic region that includes the centromere, the joint structure forming a kind of chromocenter which would resemble that found in certain stages of some other organisms, except for the fact that this complex in one genome would remain effectively separate from that of the other genome and not have recombination occurring within it. Crossing over would however occur in the four arms, and would give a four-armed map like that of a translocation heterozygote. As there would not be interference between crossings over occurring an opposite sides of a centromere, if the relations are like those in *Drosophila*, this would help to explain the abundance of multiple crossovers. It would also harmonize the cytological evidence for the existence of two chromosomes in the haploid, presented by DeLamater, with the evidence for a single complex linkage map, presented by Lederberg and his associates.

*The suggestion following the asterisk was not presented at the time of the open discussion immediately following Dr. Lederberg's paper, but was proposed the following morning, June 14, during discussion with a smaller group which included Dr. Lederberg.

POULSON: In listening to this most interesting paper it occurred to me that some of the puzzling aspects of the segregations described might be accounted for if the chromosomes of *E. coli* possess diffuse kinetochore properties rather than the highly localized type of kinetochore (the centromere) which characterizes those organisms on which most of our present genetic knowledge is based. In so far as I am aware no linkage studies have been carried out in those organisms in which diffuse kinetochores have been demonstrated. The work of the Schraders and others makes it clear that this condition prevails in a number of orders of insects and in scattered other forms. Thorough investigation of segregation and recombination in such organisms ought to be undertaken to learn in how far they follow the rules established in other organisms and in what ways they may differ.

The photographs which Dr. DeLamater showed this morning left me with the distinct impression that bacterial chromosomes may very well be possessed of diffuse kinetochores. If this should prove to be so, then your work represents the first thoroughgoing study of linkage in an organism with diffuse kinetochores. The four-armed linkage map certainly suggests, as you have emphasized, the presence of a reciprocal translocation or some mechanism of preferential segregation essentially similar in principle. Since our knowledge of the genetics and cytology of translocation heterozygotes has been based on forms with localized kinetochores it is by no means clear how the established rules apply to diffuse forms. The relationships between centromeres, crossing over, and disjunction may very well be completely different for the case of diffuse kinetochores. Perhaps the combination of your techniques with those of DeLamater will provide the answer. I realize that this is only a suggestion, but I hope it will be of value in stimulating study of the

genetics of organisms with the diffuse type of kinetochore.

WESTERGAARD: I want to discuss the problem of the very low recombination values which are obtained in these crosses. One reason may be that you have not yet found the optimal environmental conditions for sexuality here. We know both from fungi and from algae, that the sexual phase is evoked only under very special environmental conditions, often different from the optimal conditions for growth. Has any work been done to study the influence of the medium on recombination frequencies? The second possibility is that you have a mating type system in these bacteria, which is not yet quite under control. This may also explain why sexuality is confined to rather few strains. Has it been possible to rule out a mating type system in K-12?

LEDERBERG: There can be no doubt of the necessity of following the kinds of problem suggested by Dr. Westergaard. Our emphasis on formal analysis is required for just that control of the breeding technique for which *Neurospora* has been appropriately commended.

We have done a number of experiments to test the reactivity of the sexual phase to environmental changes, but no marked effects have been found. One reason, perhaps, that we have not done more in this direction is that a number of other workers here present had expressed their interest in that problem, and we were waiting to hear their results.

E. coli K-12 is recorded as a homothallic system, for no preferential compatibilities have been found in recombination experiments involving a wide range of mutants derived from K-12. In particular, no segregation of oppositional compatibility factors could be detected from persistent diploids, in contrast to the results expected from mating type mutations as reported in *Schizosaccharomyces pombe*.

Preferential compatibility would be very useful for further analysis, and is carefully looked for, especially in crosses involving new strains. Unfortunately, no encouragement is yet available from our experiments.